GENETIC VARIABILITY OF MEASLES VIRUS DURING PROPAGATION IN CULTURED CELLS

A DISSERTATION SUBMITTED TO

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SHARON LONGHURST

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LIST OF ABBREVIATION

A	adenosine
Ac	acetate
ADD-MV	acute disease-derived measles virus
AIDS	acquired immune deficiency syndrome
Arg	arginine
Asn	asparagine
ATCC	American type culture collection
C	cytosine
cDNA	complementary deoxyribonucleic acid
CD46	measles virus cell surface receptor
CNS	central nervous system
c.p.e.	cytopathic effect
dATP	2'-deoxyadenosine 5'-triphosphate
dCTP	2'-deoxycytidine 5'-triphosphate
dGTP	2'-deoxyguanine 5'-triphosphate
dTTP	2'-deoxythymidine 5'-triphosphate
DI	defective interfering particle
DMEM	Dulbecco's modification of Eagle's media
DNA	deoxyribonucleic acid
DTT	dithiothreitol
EdM	published sequence of the matrix gene of Edmonston strain of measles virus described by Bellini *et al.*, 1986
EDTA	Ethylenediaminetetra-acetic acid
ET-1	endothelin-1
F	fusion protein
FCS	foetal calf serum
G	guanine
GMEM	Glasgow modification of Eagle's media
gln	glutamine

H	haemagglutinin protein
HI	haemagglutination inhibition
his	histidine
HNT	hamster neurotropic strain of measles virus
huRS virus	human respiratory syncytial virus
i.c.	intracerebral
IMR-32	neuroblastoma cell line
IFN	interferon
IL	interleukin
I P1	Yamagata-1 virus passaged once in IMR-32 cells
I P5	Yamagata-1 virus passaged 5 times in IMR-32 cells
IPTG	isopropyl-thio-β-D-galactopyranoside
ISCOMS	immune-stimulating complexes
kb	kilobase
kDa	kilodalton
L	polymerase
leu	leucine
lys	lysine
M	matrix protein
MEM	minimal essential media
MHC	major histocompatability complex
MIBE	measles inclusion body encephalitis
MI P1	Yamagata-1 virus propagated in MRC-5 cells prior to one passage in IMR-32 cells
MI P5	Yamagata-1 virus propagated in MRC-5 cells prior to 5 passages in IMR-32 cells
MRC-5	human diploid lung fibroblast cells
mRNA	messenger ribonucleic acid
MS P1	Yamagata-1 virus propagated in MRC-5 cells prior to one passage in SK-N-SH cells
MS P5	Yamagata-1 virus propagated in MRC-5 cells prior to 5 passages in SK-N-SH cells

(mt)tRNA^{Leu}	mitochondrial leu transfer RNA
M13mp18/mp19	bacteriophage vector
NP	nucleoprotein
PBMC	peripheral blood mononuclear cells
PCR	polymerase chain reaction
P/C/V	phosphoprotein, C and V proteins from same gene
pGEMT	TA vector
p.f.u.	plaque forming units
PIP_2	phosphatidylinositol biphosphate
pro	proline
PVM	pneumovirus of mice
RC	round cell variant of measles virus
RNA	ribonucleic acid
RNP	ribonucleoprotein
S	syncytiagenic form of measles virus
SDS	sodium dodecyl sulphate
ser	serine
SK-N-SH	neuroblastoma cell line
S P3	Yamagata-1 virus passaged 3 times in SK-N-SH cells
S P5	Yamagata-1 virus passaged 5 times in SK-N-SH cells
SSPE	subacute sclerosing panencephalitis
T	thymine
TAE	tris, acetic acid, EDTA
Taq	*Thermus aquaticus* DNA polymerase
TBE	tris, boric acid, EDTA
TG2	*Escherichia coli* strain TG2
Tris	tris(hydroxymethyl)aminomethane
TRTV	turkey rhinotracheitis virus
tyr	tyrosine
U	uracil
Unk	unidentified sequence isolated during matrix gene cloning reactions of Yamagata-1 virus

Vent	*Thermococcus litoralis* DNA polymerase
Vero	African green monkey kidney epithelial cells
X-Gal	5-bromo-4-chloro-3-indoyl-β-D-galactopyranoside
YM-1	Yamagata-1 virus matrix gene sequence of virus propagated in Vero cells only, described by Wong *et al.*, 1989
YM-V	Yamagata-1 virus passaged 102 times in Vero cells
10SK	ADD-MV passaged 10 times in SK-N-SH cells
10IM	ADD-MV passaged 10 times in IMR-32 cells
10MR	ADD-MV passaged 10 times in MRC-5 cells
10VE	ADD-MV passaged 10 times in Vero cells

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DECLARATION

I hereby declare that the work described in this thesis was carried out by myself. All contributions of work or information other than my own have been acknowledged or referenced in the text. All of my work described in this thesis has neither been submitted for a degree at another institution, nor previously published.

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SUMMARY

It has been reported that the phenomenon of biased hypermutation, associated with measles virus recovered from brain tissue, can be reproduced experimentally by propagation of measles virus in cultured neuroblastoma cells. The purpose of the research described in this thesis was to evaluate this claim and establish whether biased hypermutation could be induced in measles virus from an acute infection (ADD-MV), or in an RNA virus not implicated in neural disease such as human respiratory syncytial virus (huRS virus). Accordingly, an isolate of ADD-MV and huRS virus were passaged 10 times in two neuroblastoma cell lines (SK-N-SH and IMR-32), in human lung fibroblasts (MRC-5) and in monkey kidney epithelial (Vero) cells.

The M gene sequence of huRS virus passaged 10 times in each cell line was compared with the M gene sequence of virus passaged once in the same cells. No base change was observed in the M gene of huRS virus as a result of passage in these cell lines.

A 750 base pair fragment of the M gene of ADD-MV was sequenced. Comparison of the sequence obtained from virus passaged 10 times in Vero cells with that from virus passaged once did not reveal any base changes. The M gene sequence of virus passaged 10 times in SK-N-SH cells contained one base change, at position 222, a uracil to cytosine transition. Two base changes were observed in the M gene of virus passaged 10 times in MRC-5 cells, one at position 222, as described above, and one at position 217, a cytosine to guanine base change. No changes were observed in virus passaged 10 times in IMR-32 cells when compared to that passaged once.

These data indicate that neither ADD-MV, nor huRS virus, underwent enhanced mutational events during propagation in cells of neural origin, contrary to observations reported previously by Wong, *et al.* (1989), which suggested that propagation in neuroblastoma cells induced biased hypermutation.

To determine if biased hypermutation occurred at a frequency too low to be detected by cycle sequencing, the M gene of ADD-MV passaged 9 times in IMR-32 cells, generated by PCR, was cloned into the bacteriophage vector M13. Twenty-four clones were sequenced in full. In total 21 base changes were observed, however, no more than 5 changes were seen in any one clone, and there was no directional bias in the mutations observed. However, each clone contained a large deletion at the 3' region of the gene, ranging from 1008 to 622 bases. Thirteen of the clones also contained a 56 base insertion. This insertion was shown to have high identity with human mitochondrial transfer RNA (tRNA).

The M gene of Yamagata-1 (SSPE) virus resembles that of acute measles, but contains a number of additional mutations, mainly uracil to cytosine transitions. Propagation of this virus in neuroblastoma cells resulted in additional mutations not observed in virus propagated in Vero cells (Wong *et al.*, 1989). Yamagata-1 virus was passaged in the two neuroblastoma cell lines (SK-N-SH and IMR-32), both with and without propagation in human lung fibroblast cells prior to passage in the neuroblastoma cells. Three M gene clones from passage numbers one and five in both cell lines used for this experiment, were sequenced.

Analysis of the M gene sequence of Yamagata-1 virus passaged in each cell line revealed that the virus stock contained a heterogeneous population of M gene mRNA's. Both mutated sequences and sequences identical to the M gene of ADD-MV were obtained.

Comparisons of the M gene sequence from virus passaged 5 times in neuroblastoma cells with that passaged once revealed no biased hypermutational events. However, the sequence data determined from two clones appeared to be a chimera consisting of both ADD-MV M gene sequence and Yamagata-1 virus M gene sequence. The recombination point is at position 457, a G-A mutation which is the only base change common to both ADD-MV M gene and Yamagata-1 M gene sequence.

PART I
INTRODUCTION

I. CHAPTER 1

MEASLES VIRUS

I.1.1 The *Paramyxoviridae* Family

The *Paramyxoviridae* family of viruses contain a single-stranded, negative sense, non-segmented RNA genome. Mutation appears to be the only means of generation of variation among these viruses as intramolecular recombination has not been detected in viruses with linear negative-strand genomes, and variation by the reassortment of genome subunits is not applicable as the genome is not segmented (Pringle, 1991).

This family has been classified into 4 genera: the genus *Paramyxovirus*, the genus *Morbillivirus*, the genus *Rubulavirus* and the genus *Pneumovirus* (Fig. 1). Classification was initially based on the distinctive dimensions of the nucleocapsid, the length of the envelope surface projections and the route of transmission. Paramyxoviruses and rubulaviruses are closely related biochemically because of the haemagglutinating and neuraminidase activities of their attachment glycoproteins (Kingsbury *et al.*, 1978; Matthews, 1982), whereas the morbilliviruses exhibit haemagglutinating activity only. Pneumovirus of mice (PVM) is the only member of the Pneumovirus genus exhibiting haemagglutinating activity which is restricted to mouse red blood cells (Ling and Pringle, 1989).

The classification derived on this basis has been supported by recent biochemical and molecular studies. Figure 2 illustrates the molecular organisation of the viral genome. Genes of homologous function are transcribed in the same order from a single 3' promoter site. The core protein genes are followed by the envelope protein genes with the polymerase encoding gene located furthest from the promoter. Morbilliviruses and

paramyxoviruses have 6 or 7 similarly sized open reading frames (ORF) and intergenic regions (Collins, 1991).

Figure 1. The Family *Paramyxoviridae*

Sub-family *Paramyxovirinae*

Genus *Paramyxovirus*
- Human parainfluenza virus type 1 (type species)
- Human parainfluenza virus type 3
- Bovine parainfluenza virus type 3
- Murine parainfluenza virus type 1 (Sendai virus)
- Simian parainfluenza virus type 10

Genus *Morbillivirus*
- Measles virus (type species)
- Dolphin morbillivirus
- Canine distemper virus
- Peste-des-petites-ruminants virus
- Phocine distemper virus
- Rinderpest virus

Genus *Rubulavirus*
- Mumps virus (type species)
- Avian paramyxovirus 1 (Newcastle disease virus)
- Avian paramyxovirus 2 (Yucaipia virus)
- Avian paramyxovirus 3
- Avian paramyxovirus 4
- Avian paramyxovirus 5 (Kunitachi virus)
- Avian paramyxovirus 7
- Avian paramyxovirus 8
- Avian paramyxovirus 9
- Human parainfluenza virus type 2
- Human parainfluenza virus type 4a
- Human parainfluenza virus type 4b
- Porcine rubulavirus (La-Piedad-Michoacan-Mexico virus)
- Simian parainfluenza virus 4
- Simian parainfluenza virus 41
- Mapuera virus *

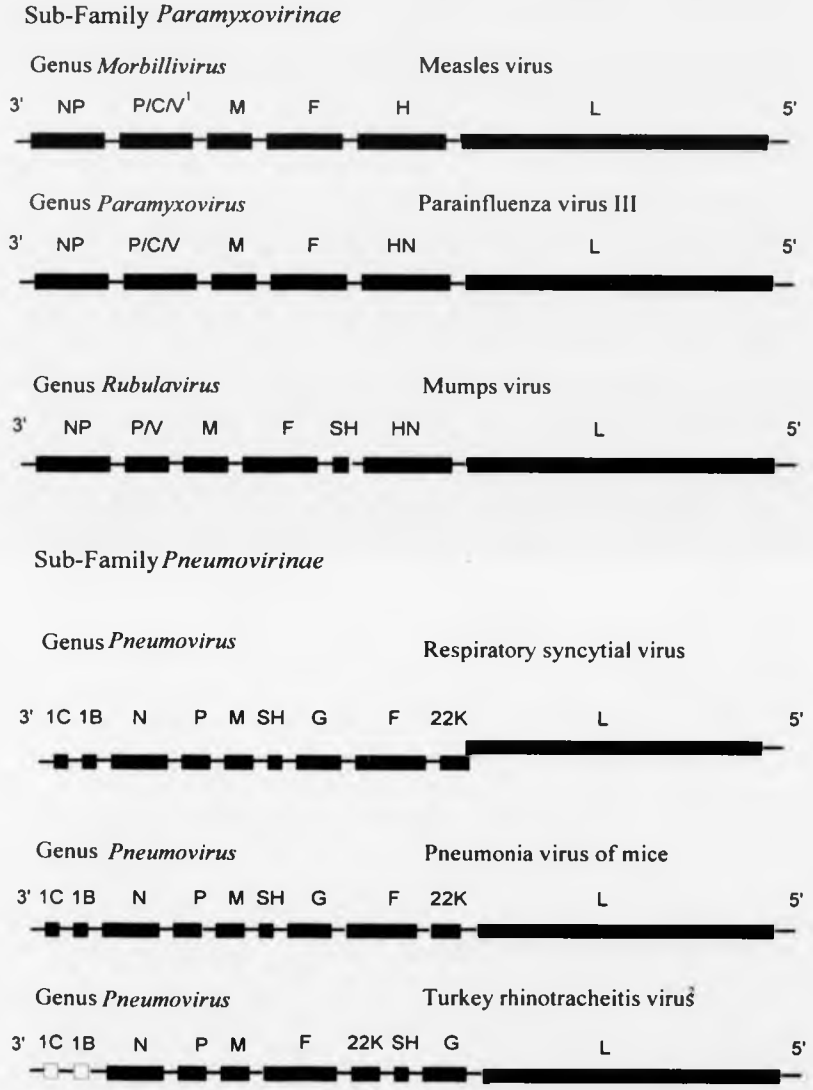
Sub-family *Pneumovirinae*

Genus *Pneumovirus*
- Human respiratory syncytial virus
- Bovine respiratory syncytial virus
- Pneumonia virus of mice (murine pneumonia virus)
- Turkey rhinotracheitis virus

Unassigned viruses in the family
- Fer-de-Lance virus
- Penguin paramyxoviruses
- Equine morbillivirus

(Murphy, *et al.*, 1995; * Henderson *et al.*, 1995)

Figure 2. Genomic organisation of viruses included in the family *Paramyxoviridae*



Notes: 1. P/C/V: multiple open reading frames of the P gene, coding for two additional proteins

2. It has not been determined whether TRTV does contain the two non-structural genes 1C and 1B. Recent results suggest that these genes are not present (personal communication Dr. Randhawa, University of Warwick, U.K.)

3. Adapted from Pringle (1995)

The pneumoviruses are unique, possessing 4 extra proteins; two non-structural proteins, IC (NS1) and 1B (NS2), a second non-glycosylated envelope protein, 22K (M2) and a small hydrophobic protein, SH, which is also present in some of the rubulaviruses. Turkey rhinotracheitis virus (TRTV) is significantly different from the other pneumoviruses in that the gene order is different and it may lack the 1C and 1B genes (Randhawa, J., University of Warwick, Coventry. Personal communication). The pneumovirus proteins are smaller than those of the corresponding proteins in viruses of the other genera and have distinct amino acid sequences (Spriggs and Collins, 1986; Barr *et al.*, 1991; Yu *et al.*, 1992a and 1992b; Chambers *et al.*, 1992). The F and L proteins do share a small amount of sequence homology with the F and L proteins of paramyxoviruses and morbilliviruses (Spriggs *et al.*, 1986; Stec *et al.*, 1991).

I.1.2 Genus *Morbillivirus*

I.1.2.1 Members of the Genus

Measles virus is a member of the genus *Morbillivirus* of the family *Paramyxoviridae*. Other members of this genus include canine distemper virus (CDV) which causes a debilitating disease in dogs; rinderpest virus (RPV) which causes disease in cattle and other large ruminants; peste-des-petits-ruminants (PPRV) which is specific to sheep, goats and other small ruminants; phocine distemper virus (PDV) which causes disease in seals, and cetacean distemper virus which causes disease in porpoises and dolphins. All viruses within this genus are closely related antigenically. They can be distinguished

easily by differential neutralisation using homologous and heterologous viruses and sera. Each virus is antigenically stable, but different strains can be isolated which show widely differing pathogenicity in the host species. Measles virus and canine distemper virus are known to persist and cause chronic neurological disease in a small proportion of cases (Barrett *et al.*, 1991).

Morbilliviruses are distinct from other members of the sub-family *Paramyxovirinae* in that they do not have any detectable neuraminidase activity, and infection with these viruses causes the formation of intranuclear inclusion bodies as part of their cytopathology.

I.1.3 Measles Virus - the Type Species

Measles is a highly contagious, self-limiting disease of childhood. The acute disease virus enters through the upper respiratory tract. The respiratory epithelium is thought to be the primary site of infection (Riley et al., 1978). The primary site of replication is not the respiratory epithelium, however, but the local lymph nodes (Cherry, 1992) Replication leads to a primary viraemia and infection of the reticuloendothelial system at multiple sites. A secondary viraemia develops 5 to 7 days after the primary viraemia. This leads to infection of endothelial cells causing enanthema, and epithelial cells causing exanthema (Ruckle and Roger, 1956). The virus is associated with leukocytes (mainly monocytes) in blood. It binds to the human membrane cofactor protein, CD46 (Naniche *et al.*, 1993a), or the membrane protein moesin if CD46 is not present on the cell surface

(Dunster *et al.*, 1995) . The primary antibody response to measles virus infection is IgM closely followed by IgG (Mathiesen *et al.*, 1990). Cell mediated immunity is expressed via CD4 and CD8 T cells. This response helps to eliminate virus that is tissue-associated (van Binnedijk *et al.*, 1989, 1990). During measles virus infection, and for several weeks after, there is some degree of immunosuppression. Infection with measles virus results in a lifelong immunity. Measles virus only infects humans and primates, but effectively, there is no animal reservoir.

The epidemiology of measles virus is determined by the contact of a susceptible individual with an infectious individual. One study estimated that 76% of household exposures of susceptibles lead to measles virus transmission, compared to 61% for varicella and 31% for mumps (Hope-Simpson, 1952). It has been suggested that, in an isolated population, a minimum of 400,000 to 500,000 susceptible individuals is required to sustain measles virus transmission (Bartlet, 1957; Black, 1966). In large populations, the rate of entry of new susceptible persons is high enough to maintain continuous measles virus transmission, so that measles is endemic. In small, isolated populations, measles may die out as the number of susceptible individuals declines before a new epidemic can occur following exposure to an incoming infectious individual.

A modified measles virus infection can develop in individuals where the virus is attenuated by antibodies (Edmonston, 1990). This is common in children who have received an immunoglobulin injection in too small amount to abort the infection. Infants with placental immunoglobulin can develop a modified measles virus infection when passive immunity is in decline and not fully protective. It may also be due to partial

failure of the measles virus vaccine. There is no IgM response in modified measles infection. The incubation period is longer, but the clinical symptoms are milder. (Katz, 1995)

Organs where complications occur due to measles virus infection include the heart (myocarditis); the respiratory tract (laryingotracheobronchitis); the gastrointestinal tract (diarrhoeal disease associated with vomiting); the haematopoietic system (thrombocytopenic purpura); and the central nervous system (sub-acute sclerosing panencephalitis).

Measles virus was first isolated in 1954 (Enders and Peebles, 1954). Characteristic cytopathic effects of measles virus infection include the formation of multi-nucleated giant cells and eosinophilic intranuclear and intracytoplasmic inclusion bodies *in-vitro*. In the 1960's the presence of structures resembling paramyxovirus nucleocapsids in neurones and glial cells of a patient with subacute sclerosing panencephalitis (SSPE) was reported (Bouteille *et al.*, 1965). SSPE is a fatal neurological disease of children and adolescents and the presence of intranuclear inclusion bodies in neurones and glial cells suggested a viral aetiology. Later, measles virus antigens and high levels of anti-measles virus antibody in the cerebrospinal fluid and serum of patients with SSPE were demonstrated (Connolly *et al.*, 1967). These observations provided strong support for the hypothesis that measles virus was the causative agent of SSPE.

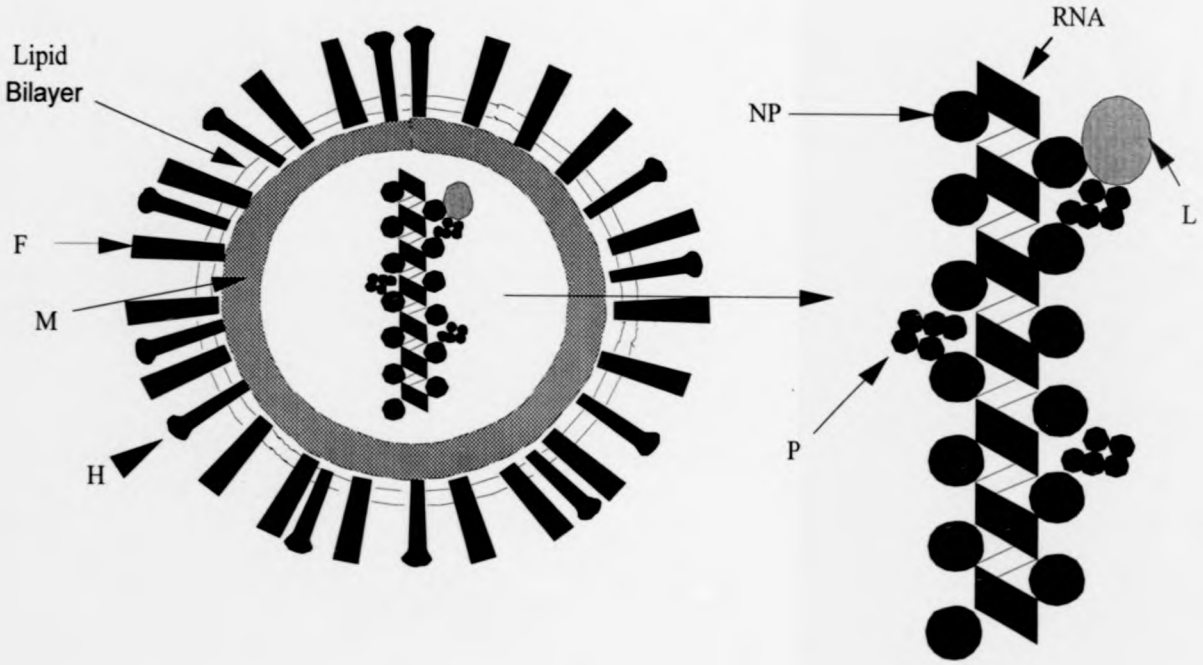
I.1.3.1 The Measles Virus Genome

Measles virions are quasi-spherical, enveloped particles with an internal helical nucleocapsid. Figure 3 is a diagrammatic representation of the structure of measles virus. The diameter of these pleomorphic particles ranges from 100 to 250 nm. The virus genome is a linear, single-stranded RNA of negative polarity. There is a 52-53 nucleotide leader sequence (Billeter *et al.*, 1984; Crowley *et al.*, 1988), followed by six non-overlapping genes (Fig. 2): nucleocapsid (N), phosphoprotein (P), which also encodes three non-structural proteins (C, V and R), matrix (M), fusion (F), haemagglutinin (H) and the polymerase or large protein (L). At the 5' end there are 36 untranscribed nucleotides constituting the trailer

I.1.3.1.1 The Haemagglutinin Protein (H)

The H protein is a type II transmembrane glycoprotein, the function of which is the attachment of virus to the cell receptor (CD46) molecule on the host cell. The H protein contains 617 amino acids with 5 potential glycosylation sites, 4 of which are used (Hu *et al.*, 1994b). These glycosylation sites are clustered in a small region in the extracellular domain of the H protein. The H protein is synthesised as monomers, most of which are converted to dimers by the formation of disulphide bonds. The enzyme responsible for this is the host-cell encoded protein disulphide isomerase which is located in the endoplasmic reticulum (ER) (Freeman, 1984). Dimerization takes place in the ER before transportation to the Golgi apparatus. Maturation of the antigenic epitopes also occurs in the ER (Hu *et al.*, 1994c).

Figure 3. A diagrammatic representation of the structure of measles virus



Key: Fusion protein (F); haemagglutinin protein (H); matrix protein (M); nucleocapsid protein (NP); phosphoprotein (P); polymerase protein (L). The nucleocapsid is helical, containing single-stranded negative sense RNA

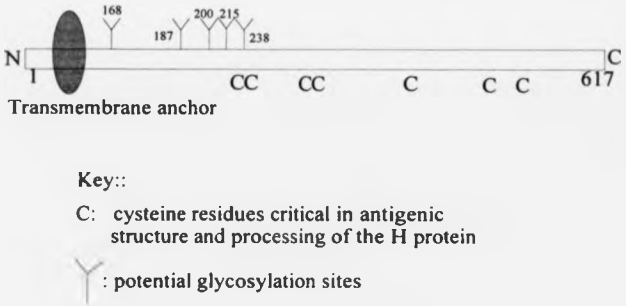
The H protein is antigenically stable (Giraudon *et al.*, 1988), and monoclonal antibodies raised to the H protein of measles virus do not cross react with the H protein of other members of the genus (Sheshberadaran *et al.*, 1983). Sequence homology of the H protein within the morbilliviruses is poor (Gerald *et al.*, 1986; Tsukiyama *et al.*, 1987; Kovammees *et al.*, 1991a), and could reflect the host specificity of the morbilliviruses. Studies have shown that the measles virus H protein has 60% homology with RPV and 37% with CDV (Gerald *et al.*, 1986; Tsukiyama *et al.*, 1987; Kovamees *et al.*, 1991a and 1991b).

There are few conserved regions within the H proteins of the morbilliviruses, therefore it is difficult to assign functional domains. There are 12 cysteine residues that are located in the ectodomain of the protein, and 7 of which are conserved within the group. These residues (287, 300, 381, 394, 494, 579 and 583) play a critical role in the antigenic structure and processing of the H protein (Fig. 4). They possibly participate in the inter- or intramolecular disulphide bonding (Hu *et al.*, 1994a).

Phenotypic differences between laboratory-adapted measles virus strains i.e., Edmonston, and recently isolated wild-type strains have been mapped to amino acids 451 and 481 of the H protein. These amino acids are thought to play a crucial role in CD46 down-regulation, hemadsorption and HeLa cell viral fusion (Lecouturier, *et al.*, 1996). Recently isolated wild-type strains of measles virus appear to lack these functions (Naniche *et al.*, 1993a & b), which may result in a stronger immune response against measles virus.

The H protein does not contain a signal peptide at the amino terminus. It has been suggested that the transmembrane region fulfils this role (Zerial *et al.*, 1987).

Figure 4: Glycosylation sites of the H protein



I.1.3.1.2 The Fusion Protein (F)

The F glycoprotein is a type I membrane protein responsible for fusion of the virion and host cell membranes. This process takes place at neutral pH and the viral nucleocapsid is released into the cytoplasm of the host cell.

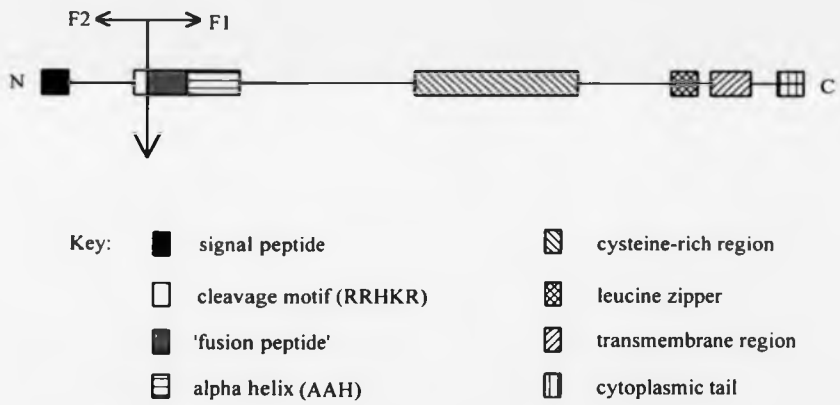
After synthesis, the precursor, F_o, protein is transported to the ER where it is glycosylated. There are 4 potential N-linked glycosylation sites, one is in the signal peptide, at Asn 6, the other three are in the F_2 region of the protein at Asn 29, 61 and 67 (Richardson *et al.*, 1986). Mutations at the Asn sites 29 and 61 produce mutant proteins that are defective in F_O protein cleavage, cell surface expression and in causing cell fusion. It seems likely, therefore, that these two sites have significant influence in the F protein conformation (Hu *et al.*, 1995; Alkhatib *et al.*, 1994b). The glycosylated F protein is transported to the Golgi apparatus, where a subtilisin-related protease cleaves the F_O

protein into two disulphide linked subunits F_1 and F_2. Identification of the cleavage site was made by sequencing the F_1 polypeptide (Varsangi *et al.*, 1985). Cleavage is initiated at a pentapeptide region comprising 5 basic amino acids (Arg-Arg-His-Lys-Arg; residues 108 to 112), and the Arg 112 residue is critical for proteolytic cleavage (Alkhatib *et al.*, 1994a). Proteolytic cleavage and transport to the cell surface occur in parallel but independently. A portion of uncleaved F protein does reach the cell surface, but released virions do not contain uncleaved F protein (Graves, 1978). This suggests that there is a positive selection of cleaved F proteins for incorporation into the virion, or that cleavage is initiated intracellularly, but continues extracellularly (Sato *et al.*, 1988). Fully processed F protein arrives at the cell membrane as an oligomer or possibly a tetramer (Sechoy *et al.*, 1987; Buckland *et al.*, 1992). F proteins are anchored in the plasma membrane by their C terminus (Blumberg *et al.*, 1985; Richardson *et al.*, 1986).

The F protein contains three hydrophobic regions (Fig. 5). The first is a signal peptide region located at the amino terminus of the F_2 subunit. This region directs the insertion of the F protein into the plasma membrane. The second hydrophobic region is the 'fusion' peptide at the amino terminus of the F_1 subunit. This region is implicated in the fusion process. The third hydrophobic region is the transmembrane anchor at the carboxy terminal of the F_1 subunit, which is responsible for anchoring the protein in the membrane. A cysteine-rich region, which is conserved in the F genes of all paramyxoviruses (Richardson *et al.*, 1986), interacts with the H protein during the fusion process (Wild *et al.*, 1994; Malvoisin and Wild, 1993). Cysteine residues 1 and 2, and 3 and 4 respectively, form disulphide loops on the F_1 subunit. It has been suggested that the H protein domain that interacts with the F protein consists of a pocket to house these

disulphide loops. There are potentially two α-helices in this protein, one is carboxy terminal to the fusion peptide (AAH), the other is amino terminal to the transmembrane region and is known as the leucine zipper (Buckland and Wild, 1989). The leucine zipper is essential for fusion (Buckland *et al.*, 1992). The function of the last 14 amino acids in the cytoplasmic tail is not fully understood. They are conserved in all morbillivirus F proteins and could be involved in the interaction of the F protein with the envelope associated matrix (M) protein.

Figure 5. Structural elements of the Fo protein which may be functionally important



Adapted from Wild & Buckland (1994)

There is at least 70% conservation of the amino acids in the F protein within the *Morbillivirus* genus (Barrett *et al.*, 1987; Buckland *et al.*, 1987; Tsukiyama *et al.*, 1988; Kovamees *et al.*, 1991a). Comparison of F protein primary sequences from different paramyxoviruses reveals a number of conserved structurally important elements which aid in the assignment of domains (Morrison and Portner, 1991).

I.1.3.1.3 The Matrix Protein (M)

The M protein is the smallest of the structural proteins of measles virus, consisting of 335 amino acids. The mRNA encoding the M protein, however, contains a long untranslated region of 425 nucleotides at the 3' end. There are two small open reading frames (ORF) following the M protein translation termination codon. The first translation initiation codon (AUG) is at position 1057 - 1059 and the second is at position 1082 - 1084. These ORFs are designated MX1 and MX2, however, attempts to determine if these ORFs are translated into proteins have been unsuccessful, thus the M gene is considered to be monocistronic. (Bellini *et al.*, 1986; Wong *et al.*, 1987).

The function of the M protein is to mediate an interaction between the envelope glycoproteins and the nucleocapsids in the cytoplasm to form new virus particles. This could be achieved by the attachment of the M protein to the nucleocapsids causing a conformational change in the M protein, so revealing regions that could interact with the cytoplasmic tails of the envelope proteins; or, the M protein could interact with the envelope glycoproteins first, so revealing regions for interaction with the nucleocapsids.

Measles virus M protein has been shown to bind nucleocapsids (Hirano *et al.*, 1993). Analysis of chimeric M proteins suggest that the conformation of the M protein is important for such an interaction, thus conformational changes in the M protein could contribute to a non-productive infection. There is no direct evidence to show interaction between the M protein and viral glycoproteins; although, in Sendai virus it has been shown that the M protein is associated with the viral glycoproteins prior to expression at

the cell surface. It was suggested that the M protein becomes associated with the viral glycoprotein during transport to the cell membrane (Sanderson *et al.*, 1993). Measles virus M protein can not be chemically cross-linked to either of the two envelope glycoproteins (Markwell and Fox, 1980), therefore, it is unknown whether it interacts with either or both of the viral envelope glycoproteins. The cytoplasmic domain of the F protein is highly conserved within the morbilliviruses, suggesting an essential function (Buckland *et al.*, 1987; Tsukiyama *et al.*, 1988). The following hypothesis has been put forward for the interaction of M protein with the F and H proteins (Wild *et al.*, 1991). First the M protein interacts with the cytoplasmic domain of the F protein. Then the F and H proteins interact, consequently it may not be necessary for the M protein to interact specifically with the H protein. Since the cytoplasmic domains of the H proteins of morbilliviruses are not conserved, it seems unlikely that this region has a specific function in assembly (Kovamees *et al.*, 1991b).

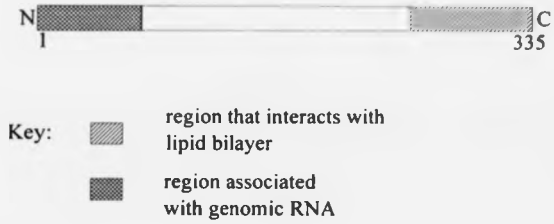
There is 67% identity at the nucleotide level and 76% identity at the amino acid level between the M genes of measles virus and canine distemper virus (Bellini *et al.*, 1986; Greer *et al.*, 1986). The C terminal one-third of the M protein is the most conserved across the paramyxovirus group and is hydrophobic in character. The predicted secondary structure for this region contains a combination of non-polar hydrophobic and β-sheet domains which suggest that the M protein may interact with a lipid environment, but may not actually span the membrane since the hydrophobic regions are considered to be too short (Bellini *et al.*, 1986; Li *et al.*, 1980). From cell fractionation studies it is clear that the M protein is associated with the plasma membrane (Bowen & Lyles, 1982; Lamb & Choppin, 1977). On the whole the M protein is hydrophobic and highly basic.

The basic nature of the protein suggests that interactions with the nucleocapsid could be via ionic attraction as the nucleocapsid is more acidic at its carboxy terminus; the highly basic amino terminus is associated with genomic RNA (Morgan *et al.*, 1984; Heggeness *et al.*, 1981) (Fig. 6). The M protein of measles virus is also associated with the cell cytoskeleton, particularly actin filaments (Bohn *et al.*, 1986). Ribonucleoprotein (RNP) cores complexed with M protein have been shown to exhibit reduced transcriptional activity, whereas RNP cores free of M protein exhibited substantially higher transcriptional activity. These results suggest that M protein may be an endogenous inhibitor of measles virus ribonucleoprotein transcription (Suryanarayana *et al.*, 1994).

RNA extracted from brain tissue of deceased SSPE patients has revealed that the M gene of measles virus persistently infecting the CNS is highly mutated. Fifty percent of the mutations seen in the M gene of measles virus isolated from an measles inclusion body encephalitis (MIBE) patient were U to C changes, a phenomenon termed biased hypermutation (Cattaneo *et al.*, 1986). Such mutations will, theoretically, effect the assembly and budding of intact viral progeny. If the recognition sites for the F protein cytoplasmic tail, and/or the nucleocapsid are mutated, then viral assembly will be diminished, thus the viral progeny cannot be released from the host cell. Thus, biased hypermutation of the M gene may play a role in the development of measles virus persistence in the CNS. It is currently suggested that biased hypermutation may occur as a consequence of propagation in neural cells (Wong *et al.*, 1989), and testing this hypothesis is one of the aims of this project. Increased activity of a double-stranded RNA adenosine deaminase enzyme has been suggested as the mechanism responsible for the U

to C transitions seen in the M gene of CNS-associated measles virus (Rataul *et al.*, 1992; Bass *et al.*, 1989).

Figure 6. Functional domains of the M protein



I.1.3.1.4 The Nucleocapsid Protein (NP)

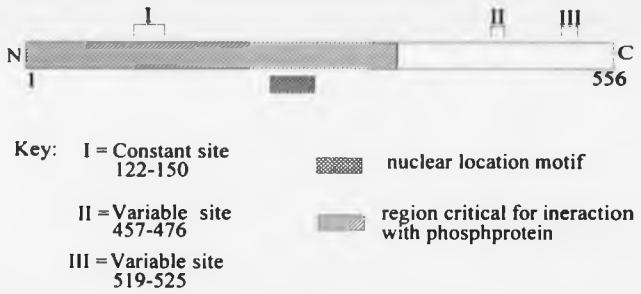
Mature NP protein is quickly and selectively incorporated into viral nucleocapsids, and is a target for interaction with the phosphoprotein, P (Gombart *et al.*, 1993). The conformational change, producing mature NP protein, does not require the participation of other viral proteins or genomic RNA; assembly of the NP protein into nucleocapsid structures can occur independently of the presence of other viral proteins or virion RNA (Fooks *et al.*, 1993).

The function of the NP protein is to encapsidate the viral RNA. Only genomic and subgenomic length RNAs which possess a 56 nucleotide leader sequence are encapsidated by the NP protein (Castaneda & Wong, 1990), suggesting an interaction between the leader sequence and the NP protein.

Nucleotide sequence comparisons of the NP protein gene of the different members of the genus *Morbillivirus* reveal regions of high and low homology. Residues 501-1215 are

strongly conserved; residues 1-500 are moderately conserved and the region 1216-1625 is weakly conserved (Rozenblatt *et al.*, 1985; Barrett and Diallo unpublished results cited in Barrett *et al.*, 1991: Morgan, 1991). Three antibody binding sites have been mapped to the NP protein; site I is a constant epitope, and is located nearer the amino terminus (residues 122-150). The variable sites (II and III) are located toward the carboxy terminus (residues 457-476 and 519-525) of the protein (Buckland *et al.*, 1989). The amino terminus and the middle region of the protein are thought to be critical for interaction with the P protein (Gombart *et al.*, 1993) (Fig. 7).

Figure 7. Structural features of the NP protein



Adapted from Buckland *et al.*, 1989; Gombart *et al.*, 1993

Measles virus nucleocapsids can be found in the cytoplasm (rough nucleocapsids) and in the nucleus (smooth nucleocapsids). Nuclear-located nucleocapsids are observed more frequently during persistent infections (Robbins *et al.*, 1983) and in neural cells from patients with SSPE (Dubois-Dalaq *et al.*, 1974b). Dingwall and Laskey (1991) have identified a bipartite nuclear location motif which consists of 2 basic amino acids, a spacer region of 10-15 amino acids, followed by a second region where 3 out of 5 amino acids are basic is nature. A similar motif has been found in the NP protein of measles

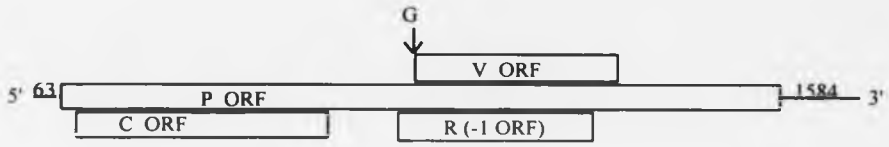
virus. The motif is located between residues 220-239 and consists of 2 arginine residues, a spacer of 9 amino acids and a region where 4 out of 9 amino acids are basic. The region is conserved in all NP proteins sequenced thus far.

The NP protein elicits a strong humoral immune response, second only to that against the H protein (Stephenson and ter Meulen, 1979), and is able to stimulate the production of specific CD4+ T lymphocytes that can protect against encephalitic disease in an animal model (Bankamp *et al.*, 1991).

I.1.3.1.5 The Phosphoprotein (P)

The P gene is 1657 nucleotides long, coding for a protein of 507 amino acids, with a molecular weight of 54-55 kDa. This gene contains two overlapping reading frames, allowing the synthesis of a shorter (186 amino acid), non-structural C protein (Bellini *et al.*, 1985; Thomas *et al.*, 1988). The C protein co-localises in infected cells with the NP protein, suggesting it may play a role in either transcription or replication. The V protein is accessed from the P reading frame by introduction of one additional G residue at position 751 during transcription, a phenomenon known as RNA editing, causing the replacement of the last 227 amino acids of the P protein, with 68 other amino acids from the V reading frame. This cysteine-rich region has been shown to specifically bind zinc (Cattaneo *et al.*, 1989a; Liston and Briedis, 1994) (Fig. 8).

Figure 8. Organisation of the P/C/V/R cistron



Adapted from Liston and Briedis, 1995

The P protein is antigenically the least conserved (Sheshberadaran *et al.*, 1986) and its nucleotide sequence is one of the most variable. It is the only cDNA, however, which cross-hybridises between all the members of the *Morbillivirus* genus (Barrett *et al.*, 1985; Barrett and Underwood, 1985).

The P protein is thought to act as a co-factor in viral transcription. It retains the nucleocapsid protein (NP) in the cytoplasm by specifically interacting with it. There are two domains on the P protein that are important in its interaction with the NP protein, the carboxy terminal 100 amino acids and the extreme amino terminal residues (Huber *et al.*, 1991; Harty and Palese, 1995).

In Sendai virus and Vesicular Stomatitis virus the amino terminus of P is highly phosphorylated and possibly interacts electrostatically with the polymerase (L) protein (Chattopadhyay and Banerjee, 1987; Vidal *et al.*, 1988). Phosphorylation is thought to be mediated by the host cell casein kinase II. Three phosphorylation sites at the amino terminal region of the P protein in measles virus have been found; Ser 86, Ser 151 and Ser 180. These phosphorylation sites will also be present in the V protein, though what effect this may have on its function has not been determined (Das *et al.*, 1995).

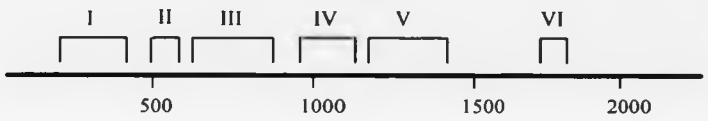
Recently, ribosomal frame-shifting of the measles virus P protein mRNA has been shown to generate a novel P cistron-encoded protein (R). Analysis of immunoprecipitated proteins from measles virus infected cells has detected a protein species consistent with the predicted molecular weight of R. Frame-shift frequency was estimated at 1.8% (Liston and Briedis, 1995).

I.1.3.1.6 The Polymerase Protein (L)

The measles virus L gene is 6639 nucleotides in length and contains only 90 untranslated nucleotides. The L protein contains 2183 amino acids and is highly basic in nature. Comparison of the L gene of measles virus and CDV has revealed approximately 67% conservation at the amino acid level (Sidhu *et al.*, 1993). Comparisons of the L gene sequence of other paramyxoviruses have been published (Blumberg *et al.*, 1988; Sidhu *et al.*, 1993). Strong sequence homology was identified in 6 blocks previously defined by Poch *et al.* (1990), which are thought to represent functional domains of the protein. These regions of homology are linked by hinge regions of low conservation. Blocks II to V have the highest amino acid conservation and are located in the centre of the protein, with divergence occurring mostly at the carboxy-terminal region of the protein (Fig. 9). The functions associated with these domains have not yet been identified. More recently the L genes of 7 strains of measles virus were sequenced, along with the L genes of phocine morbillivirus and dolphin morbillivirus. These were then compared with CDV L gene sequence, and 3 domains of homology, linked by 2 hinge regions of low conservation, were observed. It was suggested that the function of domain I was RNA

binding, domain II contained the replicase machinery and domain III contained an ATP-binding motif and protein kinase functions (personal communication, Dr. B. Rima, The Queens University of Belfast). The L protein contains 11 clusters of basic amino acids suggesting some of these might represent domains involved in electrostatic interactions with the RNA genome. It also contains two Gly-Asp-Asp (GDD) motifs (positions 3770-3779 and 4412-4420) which are thought to be characteristic of RNA-dependent RNA polymerases (Blumberg *et al.*, 1988: Komase *et al.*, 1995).

Figure 9. Conserved regions in the L protein



I to VI: putative functional domains of the L protein
Adapted from Poch *et al.*, 1990

I.1.4 Measles Virus Replication

I.1.4.1 Attachment and Entry

I.1.4.1.1 Measles Virus Receptor (CD46)

Vaccinia virus recombinants expressing measles virus H and F proteins were used to infect HeLa cells in order to screen for monoclonal antibodies which inhibited the fusion activity of the vaccinia virus recombinants. An antibody which specifically inhibited

measles virus binding and infection of human cells was shown to immunoprecipitate one or two glycoproteins with apparent molecular weights of 57 and/or 67 kDa, depending on the cell line used (Naniche *et al.*, 1992). These glycoproteins were shown to be the human membrane cofactor protein, CD46 (Naniche *et al.*, 1993a; Dorig *et al.*, 1993). CD46 is a member of the regulators of complement activation gene cluster; its biological role is to inhibit the deposition of complement proteins C3b and C4b on host cells, by acting as a cofactor for plasma serine protease factor I (Liszewski *et al.*, 1991). Multiple isoforms of CD46 (C1, C2, BC1 and BC2) are expressed on human cells and have been shown to act as a receptor for measles virus (Manchester *et al.*, 1994)

The release of N-linked oligosaccharides from CD46 by treatment with N-glycosidase F abolished the recognition of measles virus, thus N-glycan has a direct effect on the binding of measles virus to the receptor. Measles virus was unable to recognised the receptor after cleavage of the disulphide bonds, indicating that the receptor determinant on CD46 is conformation dependent (Maisner *et al.*, 1994). It has been recently confirmed that the N-linked oligosaccharides maintain the conformational protein domain that is recognised by the measles virus H protein (Maisner and Herrler, 1995).

CD46 is a type I membrane glycoprotein in which the amino-terminal two thirds are composed of 4 short consensus repeats (SCR). Each SCR is a cysteine-rich repeating domain of approximately 60 amino acids. The 4 SCRs precede a ser/thr/pro rich domain, a domain of potential heavy O-glycosylation. The extracytoplasmic region of CD46 encompassing the 4 SCRs is sufficient to act as a receptor for measles virus. The cytoplasmic and transmembrane regions of CD46 may not play a major role in the signal

for the haemagglutinin down-regulation of CD46 and / or endocytosis of measles virus (Varior-Krishnan *et al.*, 1994; Manchester *et al.*, 1995). SCR1 and SCR2 have been shown to make up the measles virus receptor determinant in CD46 (Manchester *et al.*, 1995; Buchholz *et al.*, 1996); SCR3 and 4 appear to enhance measles virus binding which suggests that one of these domains may bind the virus secondarily or that they both, in some way, influence the disposition of SCR1 and 2 (Buchholz *et al.*, 1996).

The membrane-organising extension spike protein, moesin, has also been shown to be linked with susceptibility of cells to measles virus infection (Dunster *et al.*, 1994). This protein is localised at the cytoplasmic side of the plasma membrane, but is also accessible to antibodies on the cell surface. Moesin provides an alternative route of entry for measles virus on cells that do not contain CD46 on the surface i.e. murine cells, although the uptake of virus is reduced considerably (Dunster *et al.*, 1995). Evidence has been put forward which suggests that moesin and CD46 are located in close proximity to each other and could, therefore, form a complex supporting the infection of cells with measles virus (Schneider-Schaulies *et al.*, 1995).

I.1.4.1.2 Viral Attachment and Fusion

Measles virus attaches to the cell via the H proteins affinity with the cell surface receptor, CD46 (Naniche *et al.*, 1993a). The CD46 receptor is preferentially expressed on the apical membrane, while the moesin (the alternative receptor) appears to be present in similar amount on both the apical and basolateral cell surfaces. As such, measles virus attaches to and enters epithelial cells in a polarised fashion through the apical plasma

membrane (Blau and Compans, 1995). Infection of HeLa or Jurkat cells with measles virus leads to a down regulation of the cell receptor at the cell surface (Naniche *et al.*, 1993b). Removal of the receptor results in the infected cells being more susceptible to complement mediated lysis, however, such cells will have reduced fusability, which could lead to viral persistence.

Fusion has been shown to required both the F protein and the H protein (Wild *et al.*, 1991; Cattaneo and Rose, 1993; Malvoisin and Wild, 1994). H and F proteins are processed in the cell at different rates; F arrives at the cell surface faster than H (Sato *et al.*, 1988), which suggests that the interaction of the two glycoproteins may occur at a later stage, and not during transport. The details of virus binding and cell fusion are poorly understood in measles virus. The majority of the information available for morbillivirus replication has been extrapolated from that of the other paramyxoviruses and Sendai virus.

A ternary complex consisting of H, F and the cell surface receptor is thought to be a prerequisite for fusion. The H protein may have a positioning role to optimise the conditions for fusion, however, if the F protein is present at a high enough concentration or is orientated correctly in the membrane, the H protein is no longer required (Alkhatib *et al.*, 1990). The overall function of the H protein may be to anchor the infected cell to the receptor of the target cell and then to associate with the F protein, so that it can interact with the cell membrane of the target cell.

The hydrophobic domain at the amino terminal region of the F protein is exposed after cleavage and is highly conserved among the paramyxovirus F proteins. It has been suggested that this region, the fusion peptide, could promote fusion with the target membrane (Gething *et al.*, 1978). However, it has been shown that regions of the F protein other than the fusion peptide can influence fusion activity (Richardson *et al.*, 1980; Novich and Hoekstra, 1988; Hull *et al.*, 1987). It is likely that the fusion peptide makes the initial contact with the target membrane, however, its ability to form a water-tight fusion pore has not been confirmed. The leucine zipper region on the F protein (Buckland *et al.*, 1992) is essential for fusion. It is thought to pack the α-helices together, so forming the fusion pore which can expand as more tetrameric F proteins are constructed.

I.1.4.2 Transcription and Replication

I.1.4.2.1 Transcription

Transcription is initiated on the nucleocapsid bound RNA at the 3' terminus, synthesising a 56 nucleotide positive strand leader RNA (Horikami and Moyer, 1991). RNAs destined for encapsidation are distinguished from those destined for translation by presence or absence of a transcribed leader sequence (Castaneda and Wong *et al.*, 1990). Leaderless mono- and bicistronic RNAs are associated with ribosomes and function as mRNAs which are translated. RNA species containing leader sequence are encapsidated.

Transcription occurs sequentially. It is thought that the polymerase detaches from the nucleocapsid template at the intergenic regions with increasing frequency as the distance of the gene from the promoter increases, leading to the polar accumulation of mRNAs. Messenger RNA abundance is thought to be the main determinant of protein abundance. Transcription frequencies of mRNA in infected cells relative to the NP mRNA (100%) are P (81%), M (67%), F (49%) and H (39%) (Cattaneo *et al.*, 1987; Schneider-Schaulies *et al.*, 1989). At the end of each gene the polymerase terminates synthesis following polyadenlyation and then reinitiates at the consensus sequence of the next gene without transcribing the intergenic trinucleotide (Banerjee, 1987; Banerjee and Barik, 1992). Viral mRNAs are capped and polyadenylated (Yoshikawa *et al.*, 1986). The stop region of each gene is an A/U rich sequence, followed by 4 - 7 uracil residues. This is thought to be the signal for polyadenylation of the mRNA (Udem and Cook, 1984; Yoshikawa *et al.*, 1986). The poly A tail is thought to be synthesised by a slippage mechanism in which the polymerase slips or backs up and copies the multiple uracil residues repetitively (Kolakofsky *et al.*, 1991).

Cattaneo *et al.* (1989a) showed that in measles virus-infected cells half of the P mRNA was complementary to genomic RNA and half was altered by the insertion of a single non-templated G nucleotide at position 757, giving a third open reading encoding the V protein. These data suggest that RNA editing is an additional function of the RNA polymerase. P mRNA editing is thought to occur by a stuttering or slippage mechanism similar to that propose for polyadenylation (Vidal *et al.*, 1990a, b).

Cellular proteins are also required for viral RNA synthesis (Moyer *et al.*, 1990; Horikami and Moyer, 1991; Blumberg *et al.*, 1991). Tubulin may act as either a subunit of the viral RNA polymerase (Moyer *et al.*, 1986), as an acidic activator of the RNA polymerase (Chattopahyay and Banerjee, 1988), or as an anchoring site for the transcription / replication apparatus (Hamaguchi *et al.*, 1985).

I.1.4.2.2 Genome Replication

Replication in the Morbillivirus genus has not been extensively studied. The following model for replication is based on that determined for VSV and Sendai virus. The synthesis of both genome and antigenome length RNA is coupled to their concomitant encapsidation by the NP. The first product of replication, the antigenomic RNA is encapsidated with the NP protein during its synthesis. This is then assembled into a replication competent complex and serves as a template in the synthesis of progeny viral RNA. Amplification of the progeny viral RNA and assembly into nucleocapsids serves two functions: the genomic nucleocapsids can be incorporated into mature virions, and it provides additional transcriptionally active complexes for further production of mRNA and subsequent amplification of proteins that are required for virion assembly (Choppin and Compans, 1975; Choppin and Scheid, 1980). *De novo* protein synthesis has been shown to be a requirement for replication (Carlsen *et al.*, 1985).

The P-L transcription complex and the availability of the NP protein is thought to regulate the transition from transcription to replication (Banerjee, 1987; Blumberg *et al.*, 1981b). As only leader-containing RNA is replicated, it follows that there must be some

recognition site for the interaction of the NP protein to the RNA. The first 14 bases at the 5' end of the leader sequence maps to the site for the initiation of nucleocapsid assembly (Blumberg *et al.*, 1983). A minimal model for the switch from transcription to replication has been put forward for VSV (Blumberg *et al.*, 1981a & b; Kolakofsky and Blumberg, 1982; Arnheiter *et al.*, 1985; Banerjee, 1987). Insoluble NP protein complexes with the P protein producing a soluble substrate for encapsidation. The RNA polymerase catalyses the binding of NP to the leader RNA, releasing P. Encapsidation with NP coupled with further RNA synthesis masks the termination signals at each gene boundary, preventing termination and mRNA processing, thus, yielding full length assembled nucleocapsids.

I.1.4.3 Assembly and Release of Viral Progeny

The sequence of events in the assembly of measles virus proteins at the plasma membrane are: (i) transport vesicles carrying H and F proteins from the Golgi apparatus fuse with the plasma membrane. It is likely that these proteins arrive at the plasma membrane in separate vesicles as the maturation and transport of the F protein is faster than that of the H; (ii) newly delivered proteins are probably distributed randomly in the cell surface and are mobile, so they can diffuse laterally in the plane of the membrane; (iii) the M protein becomes associated with the plasma membrane, probably by interaction with the cytoplasmic domain of the F protein; (iv) viral glycoproteins are localised in specific patches of the plasma membrane containing M protein, from which host-cell surface proteins are excluded; (v) nucleocapsids associates with the modified areas of the plasma membrane, probably by interaction with the M protein, presumably triggering the

budding process (Misek *et al.*, 1984; Matlin & Simons, 1984; Pfeiffer *et al.*, 1985; Rindler *et al.*, 1984).

Virions are formed by a process of outfolding, or budding, at the cell surface (Compans *et al.*, 1966). The budding process of measles virus starts with a gradual elevation of strand-like structures from the plasma membrane, leading to larger protrusions which eventually progress into spherical virus particles (Bohn *et al.*, 1983). Although most cellular membrane proteins are effectively excluded from the envelopes of budding virus particles, it has been shown that the cellular cytoskeleton, particularly actin filaments, is involved in virus budding, and that budding itself is possibly the result of a vectorial growth in actin filaments (Bohn *et al.*, 1986). Measles virus release has been shown to be polarised i.e., it only occurs at the apical surface of epithelial cells (Blau and Compans, 1995).

I.1.5 Measles Virus Immunobiology

I.1.5.1 Humoral Response

Antibodies to the measles virus are first detectable with the onset of the rash (Bech, 1959). The initial immunoglobulin isotypes produced are IgM and IgA, followed by the IgG. The IgG response is mediated by two isoforms of this class of immunoglobulin, namely IgG1 and IgG4 (Mathiesen *et al.*, 1990; Ehrnst, 1978). IgG1 is efficiently transported across the placenta. Plasma levels of IgE also rise, but there is no evidence that these immunoglobulins are measles virus-specific (Black, 1989; Griffin *et al.*, 1985).

Infection with the measles virus leads to life-long immunity but the mechanism of the longevity of the antibody response is not known.

Maternal antibodies are important for protection of new-borns. Such passive immunity wanes gradually as the infant catabolises maternal antibodies. The majority of infants are susceptible to measles virus infection by the age of approximately 12 months in developed countries, and 9 months in developing countries (Black, 1989; Halsey *et al.*, 1985). The average age at measles virus infection depends on the age at which maternal antibody is lost and on age-related changes in frequency of contact with other persons. In developing countries measles virus infection occurs in younger children due to the higher birth rates, more crowded living conditions and the earlier loss of maternal antibodies (Black *et al.*, 1986).

Antibodies are capable of preventing virus-induced membrane fusion and lysis of infected cells. They are also capable of modulating viral antigen expression at the cell surface and suppressing intracellular synthesis of virus protein and RNA *in-vitro*. Therefore, antibodies may play a role in controlling viral replication and also in establishing persistent infections (Ehrst, 1975; Barrett *et al.*, 1985; Fujinami and Oldstone, 1979; Schneider-Schaulies *et al.*, 1992; Rammohan *et al.*, 1983).

I.1.5.2 Cell-Mediated Responses

Non-specific and specific cellular immune responses may be activated during systemic measles virus infections. Interferon (IFN) α and β levels in plasma are not elevated (Shiozawa *et al.*, 1988) and natural killer cell activity is lower in infected individuals when compared to non-infected controls (Griffin *et al.*, 1990a).

Proliferation of peripheral blood mononuclear cells (PBMC) and elevation of plasma levels of CD8 and IFN-γ are highest during the rash (Griffin *et al.*, 1989; 1990b). This is consistent with the likely role of MHC class I-restricted (CD8) cytotoxic T cells in clearance of the virus from its sites of replication (van Binnendijk *et al.*, 1990). More prolonged increase occurs in levels of soluble interleukin 2 (IL-2) receptor, a product of IL-2 stimulated T cells (CD4), and neopterin, a product of IFN-γ stimulated macrophages, and β_2-microglobulin (Griffin *et al.*, 1989; 1990b; 1992). Long after recovery from measles virus infection, memory CD8 cytotoxic T cells can be demonstrated in PBMC (Lucas *et al.*, 1982; van Binnendijk *et al.*, 1989).

CD4 T cells are also activated in response to measles virus infection. These cells also proliferate during the rash (Ward *et al.*, 1989) and soluble CD4 becomes elevated and remains so for several weeks (Griffin and Ward, 1993). Measles virus specific MHC class II-restricted cytolytic CD4 cells are generated (van Binnendijk *et al.*, 1989; Jacobson *et al.*, 1984, 1989) and may contribute to virus clearance from tissue, however, CD4 T cells probably exert most influence by secreting cytokines. Measles virus proteins

that stimulate the proliferation of MHC class II-restricted CD4 cells include H, N, P and F (Rose *et al.*, 1984; van Binnendijk *et al.*, 1992; Bellini *et al.*. 1981; Ilonen *et al.*, 1990; Jacobson *et al.*, 1989).

The immune suppression that accompanies measles virus infection is thought to contribute to the susceptibility to secondary infections that account for most of the morbidity and mortality associated with measles virus infection (Beckford *et al.*, 1985). Possible mechanisms to explain how immunosuppression may occur include: (i) virus killing of infected lymphocytes, thus no secretion of cytokines and no activation of the immune system occurs; (ii) virus effects on monocytes; (iii) a direct viral effect on lymphocyte functioning. It is possible that all three mechanisms play a role in immune suppression.

I.1.6 Measles Virus Vaccines

The measles vaccine (Edmonston strain) in use currently is an attenuated live vaccine, administered subcutaneously, between the ages of 9 to 20 months. The antibody response is good, primarily IgG1 (Mathiesen *et al.*, 1990), provided it is administered after maternal antibody levels have declined (Albrecht *et al.*, 1977). Antibody levels induced by vaccination are lower than those seen in natural infections, but a response can still be measured 10-15 years after vaccination, in the absence of boosting infections (Black, 1989; King *et al.*, 1993; Markowitz *et al.*, 1990).

Cell-mediated immune responses have not been extensively studied, but are thought to be comparable to that of a natural infection. Antigen-presenting cells are found to be infected and able to process measles virus proteins in both MHC class I and II restricted cells, thus CD8 and CD4 T cells should be stimulated.

A high titre version of the Edmonston-Zargreb vaccine containing $>10^{4.7}$ pfu/dose was shown to elicit an immune response in individuals containing maternal antibodies. Therefore, in 1992, clinical trials using this vaccine were conducted in developing countries. This type of vaccine was thought to be useful in such countries as children come into contact with measles virus at a much earlier age, when maternal antibodies are still circulating and the standard vaccine is ineffective. A few children who received the high-titre vaccine were observed to die of unrelated diseases, such as pneumonia, diarrhoea and parasitic infections, at later times, perhaps as a consequence of immune suppression induced by the vaccine, allowing the secondary infections to become established (Weiss, 1992). Due to the slight excess of cumulative deaths in vaccinated children the trials were terminated (Garenne *et al.*, 1991).

Inactivated measles vaccine, developed prior to the use of the attenuated live virus vaccine, was found to elicit measles virus neutralising and HI antibody, but little antibody to N and none to F (Carter *et al.*, 1962; Norrby *et al.*, 1975;). Antibody response and protection by this vaccine was short-lived. Individuals receiving the inactivated vaccine were likely to develop an atypical form of measles virus, which can be life-threatening after exposure to wild-type virus (Cherry *et al.*, 1972; Brodsky, 1972).

Live attenuated vaccine works well, but periodic outbreaks of measles infection do still occur in developed countries even where there is a high vaccination rate. Improper storage of the vaccine may be responsible in part, however, data also suggests that measles virus may spread among vaccinated as well as unvaccinated individuals without causing major symptoms (Chen *et al.*, 1990; Pedersen *et al.*, 1989). New generation vaccines may help to overcome this problem. Such vaccines may include live vectors e.g. vaccinia virus and adenovirus presenting different structural proteins of measles virus (Taylor *et al.*, 1991b; Wild *et al.*, 1992; Alkhatib *et al.*, 1990), or genetically engineered newly attenuated measles virus. Novel non-infectious vaccines, comprising viral glycoproteins incorporated into subunit structures, known as immune-stimulating complexes (ISCOMS), have shown promise (de Vries *et al.*, 1988). Chimeric peptides of measles virus F protein and promiscuous T-cell epitopes, which bind several forms of MHC class II molecules, have been evaluated for their ability to elicit an immune response in mice. The results indicated that the promiscuous T-cell epitopes can be used with immunogenic peptides to produce highly immunogenic preparations capable of overcoming haplotype-restricted responses (Lairmore *et al.*, 1995). Vaccinia virus recombinants have been shown to greatly improve the immune responses in mice if two protein genes are inserted. Vaccinia virus recombinants containing only the F protein gene only elicit a poor immune response. However, if the NP gene is also inserted, the response is increased by more than 75% (Wild *et al.*, 1992).

Adenovirus recombinants containing only the NP gene have been assayed for their ability to elicit an immune response in mice. Such recombinants have been shown to induce a

humoral response to NP protein; an MHC class I restricted antigen-specific cytotoxic T cell response: and protection against challenge with the homologous measles virus strain in mice (Fooks *et al.*, 1995). This type of vaccine overcomes the maternal antibody barrier and can be used in immunocompromised patients (de Vries *et al.*, 1988). However, there is likely to be an immune response elicited against the adenovirus proteins present.

I.CHAPTER 2

THE ASSOCIATION OF MEASLES VIRUS WITH NEUROLOGICAL DISEASE

I.2.1 Subacute Sclerosing Panencephalitis and Other Progressive Neurological diseases

Measles virus associated with the central nervous system (CNS) can lead to acute or chronic disease progression. Acute measles encephalitis is a rare complication of acute measles virus infection (Aarli, 1974) which can occur during the infection or shortly after recovery. It is thought to be the result of virus-induced autoimmune reactions (Schneider-Schaulies *et al.*, 1995). Sub-acute sclerosing panencephalitis (SSPE) and measles inclusion body encephalitis (MIBE) are very rare complications associated with measles virus persistence in the CNS. SSPE can present many years after the acute disease. It is characterised by progressive cerebral dysfunction. MIBE affects immunocompromised patients, such as those with leukaemia or AIDS (Hughes *et al.*, 1993; Aicardi *et al.*, 1977). It has a variable incubation time and presents as a non-specific encephalitis.

As early as 1965 a paramyxovirus was the suspected causative agent of SSPE. Electron micrographs of brain tissue of an SSPE patient showed paramyxovirus-like nucleocapsids within the cells (Boutille *et al.*, 1965), subsequently confirmed by others (Dayan *et al.*, 1967; Katz *et al.*, 1969).

The mechanisms which define how measles virus changes from a lytic, self-limiting infection, to that of a non-productive, persistent infection are not clear. There is a latent period of 6 to 7 years between the initial acute measles virus infection and the onset of the first neurological changes seen in SSPE patients. There is a 2- to 3-fold excess of SSPE in males, with children from rural areas at greater risk. SSPE tends to develop in children who contract acute measles at an early age; i.e. an average age of 2 years (Modlin *et al.*,

1979). The earliest signs of SSPE consist of intellectual deterioration and behavioural changes, followed by progressive neurological impairment leading to cortical blindness, dementia and finally death. The majority of patients die within 1 to 3 years of the onset of the disease.

The incubation period from acute measles virus infection to the first signs of MIBE can vary from 1 to 6 months, and is confined to patients with immune deficiency. This disease is less well defined than SSPE. It is a haemorrhagic leukoencephalitis with intranuclear inclusion bodies which contain paramyxovirus nucleocapsids.

Elevated measles antibody titres in the serum and cerebrospinal fluid of SSPE patients is observed, especially to the NP protein and the P protein and, to a lesser extent, the two surface glycoproteins, H and F. Measles virus antigens can also be detected in the brain of SSPE patients by immunofluorescence (Freeman *et al.*, 1967; Connolly *et al.*, 1967). Inflammatory lesions in both the white and grey matter of the brain, and occasionally the cerebellum and spinal cord, can be seen, and demyelination is also seen to varying degrees. Reduced levels of viral envelope protein mRNA, probably due to enhanced attenuation of transcription at each gene junction, is also observed. Expression of envelope proteins seems to be diminished by specific translational inhibition (Schneider-Schaulies *et al.*, 1993; Yoshikawa and Yamanouchi, 1984; Miller and Carrigan, 1982).

Infectious virus can be recovered from the brain tissue of SSPE patients, by co-cultivation with cells permissive for growth of measles virus (Homma *et al.*, 1982; ter Meulen *et al.*, 1972). Measles virus found in the brains of SSPE patients is non-productive and non-

lytic; no detectable extracellular virus particles are produced. However, virus recovered by co-cultivation (usually called SSPE virus) is generally productive and lytic in tissue culture. Attempts to recover virus from brain tissue by co-cultivation have a failure rate of approximately 80%, thus the rescued virus may represent either a minor population of the virus present, or a new mutant that occurs during the co-cultivation procedure. Nucleotide sequence comparisons indicate, however, that some so-called SSPE viruses i.e., Halle, Mantooth and Horta-Barbosa (I.3.2. Table 1) are in fact derived from standard strains probably as a result of cross-contamination occurring in the laboratory.

Occasionally, a non-lytic SSPE virus may be rescued. Non-lytic virus remains cell-associated and little or no infectious virus is produced (Burnstein *et al.*, 1974; Ueda *et al.*, 1975; Homma *et al.*, 1982). Some non-lytic SSPE viruses recovered by co-cultivation can cause neurological disease when used to infect mice (Homma *et al.*, 1982), and this type of virus may reflect more accurately the characteristics of the virus in the brain. An example of such a virus is the Yamagata-1 strain, which is the subject of extensive characterisation in this thesis.

I.2.2 Molecular Biology of SSPE Viruses

I.2.2.1 Experimental Material and Viral Strains

Due to the difficulty in isolating live virus the majority of the work carried out on SSPE viruses has been done by extracting RNA from the brain tissue of deceased patients. Several genes of a variety of different SSPE virus strains have been sequenced, in

particular the M, F and H, and to some extent NP. Comparing the sequences obtained with that of a consensus sequence for acute measles virus, nucleotide changes were observed, particularly in the M gene (Cattaneo *et al.*, 1986, 1988a, b; Wong *et al.*, 1989; Ballart *et al.*, 1991). Interpretation of the significance of nucleotide differences between brain derived (SSPE and MIBE) and other non-defective, tissue culture adapted measles virus has been hampered by the fact that the non-defective viruses can be grouped into several different genotypes (Cattaneo *et al.*, 1989b; Taylor *et al.*, 1991a; Rima *et al.*, 1995b). In order to determine beyond doubt that nucleotide differences between SSPE viruses and acute disease virus are significant, the lineage of the acute virus which initially infected the patient would be required for sequence comparisons. Unfortunately, the circulating lineage at the time of the acute infection is rarely known and can only be surmised, therefore, most studies have used a consensus sequence for the acute virus, as such, some of the conclusions may be misleading.

Analysis of different areas of the brain of SSPE patients has revealed both wild-type-like and variant sequences. The distribution of the virus variants, determined by sequence analysis and *in-situ* hybridisation, suggested that the mutated viruses expanded clonally throughout the brain (Baczko *et al.*, 1993). Five different measles virus variants were observed on the basis of the major sequence changes. These 5 variants appeared to have evolved from a progenitor by at least 5 independent hypermutation events. Variants with M gene sequence corresponding to group B3 were found in all brain regions tested. Variants belonging to group B4, which appear to have been derived from the B3 variants, were only detected in the right frontal lobe of the brain. Variant virus B2 has apparently undergone at least one hypermutation event in which 13 uracil residues of the B1 virus

variant were replaced by cytosine residues. The relationship between B1, B2 and B3 was not linear. An hypothetical intermediate, termed Bx, was required to develop a clonal link between groups B2 and B3. Although wild-type-like intact genes were found, functional M protein could not be detected (Baczko *et al.*, 1993).

The nucleotide sequence of the M gene of two wild-type strains of measles virus (JM, isolated from Bethesda, USA in 1977, and CM, isolated from USA in the late 1970's), both of which are distinct from vaccine strains of measles virus, has been determined (Bazcko *et al.*, 1991). These two wild-type viruses have been placed in separate genotypic groups (I.3.2). The sequences obtained were compared with the sequence of the M gene of an SSPE virus (Case B) and that from an MIBE patient. It was found that the acute virus strain, JM, was very similar to the SSPE virus (case B), whereas the acute strain, CM, was similar to the MIBE virus. The number of differences between the M gene sequence of the SSPE virus and JM resulted in only 6 amino acid changes between the two strains. The number of differences between JM and the SSPE virus was so small that it probably represents a divergence no greater than that observed between virus strains within one group. These results suggest that there is little or no difference between wild-type and SSPE viruses of the same genotype. However, the differences observed between the MIBE M gene and that of CM revealed many nucleotide exchanges, with a bias for uracil (U) to cytosine (C) transitions (biased hypermutation). After correcting for the U-C transitions the mutation rate was only 0.9%. This suggests that an acute disease virus from the CM lineage may have caused the initial measles virus infection in the MIBE patient, implying that the biased hypermutation events may be

important in altering the phenotype of the virus from a lytic infection to a cell associated, persistent infection (Baczko *et al.*, 1991).

Eighty percent of the nucleotide differences found in the M gene of the Biken strain of SSPE and the Nagahata strain of acute measles virus were U-C transitions. It was presumed, therefore, that the Nagahata strain of measles virus was the acute progenitor of the Biken strain of SSPE (Wong *et al.*, 1991). Such biased hypermutation accounted for all but one of the predicted amino acid substitutions. As a result of these mutations the Biken M protein loses conformation-specific epitopes that are conserved in the acute virus. Such conformational epitopes are also missing in the Niigata and Yamagata SSPE viruses (Wong *et al.*, 1991).

I.2.2.2 Theoretical Mechanism of Biased Hypermutation

Sequence data from the matrix gene of an MIBE case revealed that 50% of the U residues were changed to C and the phenomenon was termed biased hypermutation. It was hypothesised that the mutations may confer a selective advantage to the virus, so guaranteeing their survival (Cattaneo *et al.*, 1988a). Wong *et al.* (1989) attempted to reproduce this phenomenon *in-vitro*. One aliquot of an SSPE virus (Yamagata-1) was propagated in a non-neural cell line (Vero) and another aliquot was propagated in a neuroblastoma cell line (IMR-32). The neuroblastoma cell-passaged virus contained a cluster of additional U-C transitions that were not observed in the non-neural cell passaged virus. Ninety percent of these additional changes were identical to the hypermutated nucleotides seen in the M gene of the MIBE patient discussed above. The

random nature, apparent host dependency and specific location of these mutations suggested that they might be caused by an extrinsic biased hypermutational activity rather than intrinsic polymerase errors (Wong *et al.*, 1989).

A possible explanation for the observed clustering of the U-C transitions in the MIBE case is that transcription of the genome is carried out by a measles virus RNA polymerase complex which is prone to non-selectively incorporate U or C residues when copying adenosine (A) (Cattaneo *et al.*, 1986). Another hypothesis is that biased hypermutation is due to the activity of a cellular enzyme which causes the deamination of adenosine residues in dsRNA (Bass *et al.*, 1989). This enzyme, double-stranded RNA adenosine deaminase (DRADA) was originally found in *Xenopus* eggs and several mammalian cell lines (Bass and Weintraub, 1987; Wagner and Nishikuro, 1988). DRADA activity has been found to be present in a wide range of tissues and cell types, regardless of cell differentiation, and is located in the nucleus. It is thought to be a housekeeping enzyme, modifying dsRNA prior to degradation (Wagner *et al.*, 1990). The enzyme unwinds ds RNA, deaminating A residues to generate inosine (I). Inosine can then direct the incorporation of C into the positive strand mRNA, thus expressing a U-C transition (Bass *et al.*, 1989). Two hypotheses have been suggested to account for the presence of dsRNA during transcription of the viral genome. The first is that nascent transcripts could occasionally remain base paired with the template RNA ("collapsed" transcription), which would lead to the eventual arrest of transcription. Alternatively, localised double-stranded regions could occur due to the hybridisation of measles virus mRNAs to genomic RNA. Except for the similarly biased nature of the mutations seen in measles virus and the observation DRADA activity exists, there has been no evidence that links

these two phenomena. However, the level of activity of this enzyme has been found to be higher in IMR-32 cells (a neuroblastoma cell line) than in Vero cells (monkey kidney epithelial cells) (Rataul *et al.*, 1992).

Measles virus replicates in the cytoplasm, so how might genomic RNA of measles virus be exposed to this nuclear enzyme? The nuclear membrane has been shown to breakdown during meiosis and mitosis, which may lead to the leakage of the enzyme into the cytoplasm, however, neurones do not divide after maturation and, as this is the site of infection during SSPE and MIBE, it is unlikely that this explanation is tenable. Alternatively the integrity of the nuclear membrane may be disrupted at a late stage of infection. Cells infected with acute measles virus do show nucleocapsid structures in the cytoplasm (fuzzy in appearance) and the nucleus (smooth in appearance) (Dubois-Dalaq *et al.*, 1974b). Although post-mortem brain sections from SSPE patients do show a greater number of nuclear located nucleocapsids, there is no evidence that these smooth nucleocapsids are active in RNA replication and are able to pass on genetic changes to progeny virus. Measles virus RNA can be recovered from the nuclear fractions of measles virus infected cells, and as DRADA is a nuclear associated enzyme it is possible that the viral RNA could be exposed to the enzyme (Carter *et al.*, 1973; Schluederberg and Chavanich, 1974).

I.2.2.3 Matrix Protein of SSPE Viruses

The conformation of the M protein is important if it is to successfully interact with the nucleocapsid prior to viral budding (Hirano *et al.*, 1993). Mutations in the amino terminal, carboxy-proximal or carboxy-terminal regions of the M protein seem to abolish nucleocapsid binding. M protein from the Biken strain of SSPE cannot interact with nucleocapsids, although its progenitor's M protein (Nagahata) can bind nucleocapsids from the acute Nagahata infection, and that from the defective virus (Biken). The interaction of acute viral M protein with SSPE derived nucleocapsid, however, is unstable. As such the SSPE virus M protein appears to be functionally different compared to its progenitor acute measles virus strain (Hirano *et al.*, 1992a, b; Suryanarayana *et al.*, 1994). The M protein in acute measles virus is thought to interact with the nucleocapsid and the two surface glycoproteins in order to aid the release of the viral progeny. The failure of the M protein to interact with the nucleocapsids, in SSPE viruses may prevent the viral progeny from being released from the host cell, thus explaining the lack of viral progeny seen in persistent infections of measles virus in the CNS.

There is a reduced antibody response to the M protein of measles virus in patients with SSPE (Hall *et al.*, 1979; Wechsler *et al.*, 1979). This is possibly due to a reduction in the amount of protein produced in infected cells. In SSPE cell lines, which have been shown to produce only cell-associated virus, mRNA specific for M protein was observed but it was not translated into functional protein (Carter *et al.*, 1983). The lack of functional M

protein could be due to premature termination of translation due to nonsense mutations in the mRNA, loss of a translation initiation codon or possibly rapid degradation of any protein that is produced (Enami *et al.*, 1989; Ayata *et al.*, 1991). An alternative explanation is that translation of the M gene is aberrant due to synthesis of M mRNA exclusively in a P-M bicistronic form (Cattaneo *et al.*, 1986). Bicistronic mRNA is unable to direct protein synthesis form the downstream cistron (Wong and Hirano, 1987). As such, this transcriptional defect effectively blocks protein synthesis from the M cistron. Support for this explanation comes from the observation that Vero cells persistently infected with the hamster neurotropic strain of measles virus show no virus production, and display a predominance of bicistronic M-F transcripts. No F protein could be detected, probably due to the block of translation of the downstream cistron (Hummel *et al.*, 1994). Matrix protein itself has been shown to down-regulate transcription of measles virus ribonuclear protein (Suryanarayana *et al.*, 1994).

I.2.2.4 Fusion Protein of SSPE Viruses

Mutations were found in the F gene of SSPE viruses. Such mutations are predicted to cause truncations, elongations or non-conservative amino acid replacements (Schmid *et al.*, 1992). It was suggested that alterations in the cytoplasmic domain of the F protein could hinder the interaction between the M protein and F, thus preventing viral release. Truncation of the F protein intracellular domain (33 amino acids) in SSPE viruses does not impair the fusion activity of the protein, however, it may reduce functional interaction

with the M protein, thus, hindering viral assembly and budding (Cattaneo and Rose, 1993).

I.2.2.5 Haemagglutinin Protein of SSPE Viruses

Analysis of the sequence of the H protein gene of SSPE viruses has suggested that the proteins may be defective in intracellular transport, oligosaccharide modification, dimerization and fusion (Cattaneo and Rose, 1993). Fusion activity is maintained in SSPE viruses, although it occurs at a greatly reduced rate. This is primarily due to the reduced expression of functional H protein (Billeter *et al.*, 1993). The neurovirulence of various neutralising monoclonal antibody escape mutants of the rodent adapted CAM strain of measles virus has been correlated with specific protein changes between amino acid residues 368 and 396 in the H protein (Liebert *et al.*, 1994). Measles virus replicating in the brain at the terminal stage of infection is typically defective in M protein and the two surface glycoproteins. M protein appears to be dispensable altogether, whereas partial preservation of F and H function is required. Limited cell fusion will enable the virus to transfer replicating ribonuclear proteins from cell to cell, while only minimal amounts of viral proteins are exposed at the cell surface, thus hiding the infection from immune surveillance.

I.2.2.6 Nucleoprotein and Phosphoprotein of SSPE Viruses

Nucleotide sequence comparisons indicate that amino acid changes accumulate in the carboxyl terminus of the N protein and the amino terminus of the P protein. However, these changes are not thought to significantly alter the products (Cattaneo *et al.*, 1989a). The hamster neurotropic (HNT) strain of measles virus was found to have 5% amino acid changes in the N protein, 8.1% in the P protein, 2.9% in the V protein and 7.0% in the C protein when compared to the progenitor strain. Editing of the P mRNA of the neurotropic strains was found to shift from transcripts with the single guanine (G) insertion, which produces the V protein, to transcripts with greater than two G's inserted. The altered editing phenotype of the persistent measles virus strain could be a consequence of mutations in P, L or both, or related to changes in the recognition motif. Curran *et al.* (1991) suggested that the V protein may play a role in paramyxovirus persistence. They found that increased V protein expression resulted in a decrease in the replication of a defective interfering Sendai virus genome. The altered editing distribution of HNT in the study by Vanchiere *et al.* (1995) would, therefore, be expected to favour viral replication since the expression of the V protein might be reduced by the decrease in P mRNAs with 1 G inserted. By relieving the inhibitory effects of V under limiting levels of L, viruses with altered editing distributions may have a selective advantage in the persistent state (Vanchiere *et al.*, 1995).

In vivo experiments using Lewis rats with acute encephalitis and subacute encephalitis have shown strongly attenuated transcription of mRNA along the measles virus genome. A steep gradient of measles virus transcripts was found in the brain tissue. This was probably due to strongly attenuated transcription of mRNAs along the viral genome, representing particularly low levels of transcription of the glycoprotein genes. The transcriptional restriction of the glycoprotein-specific mRNAs was seen as soon as measles virus-specific mRNA was detectable in infected brains. This phenomenon was independent of the incubation time after infection, suggesting that brain-specific host cell factors may primarily interfere with measles virus gene expression. Only N and P proteins were consistently translated (Schneider-Schaulies *et al.*, 1989). Fusion and H protein mRNA can be found only in low levels in the brain tissue of SSPE patients, compared to lytically infected cells (Baczko *et al.*, 1986). Overall, measles virus specific transcription is substantially reduced in brain tissue from SSPE patients (Schneider-Schaulies *et al.*, 1992, 1994b).

I.2.2.7 Summary

In general, the nucleotide sequences of the individual genes of the SSPE viruses resemble those of the acute measles virus strain that is thought to be its progenitor (Wong *et al.*, 1991). In many cases the differences between the acute measles virus strain and the SSPE virus are U-C transitions, a phenomenon termed biased hypermutation. Such mutations are thought to confer a selective advantage for the survival of the measles virus in the CNS (Wong *et al.*, 1991; Cattaneo *et al.*. 1988a, b). It has been hypothesised that the U-C transitions could be the result of the activity of a nuclear located DRADA

enzyme (Bass *et al.*, 1989). Measles virus specific transcription is reduced in brain tissue from SSPE patients (Schneider-Schaulies *et al.*, 1992, 1994b). Cell lines derived from SSPE brain tissue produce only cell associated virus. This could be as a result of the mutations observed within the SSPE virus genomes, in particularly the M gene. Little or no M protein can be detected in SSPE virus infections. As this protein is required to orchestrate viral assembly and budding, reduction in its production is likely to result in a reduction of viral progeny release. Mutations in the F protein, particularly the cytoplasmic domain which is thought to interact with the M protein during viral assembly, will also have an effect on the release of viral progeny (Cattaneo *et al.*. 1986; Cattaneo and Rose, 1993; Schmid *et al.*, 1992; Enami *et al.*, 1989; Ayata *et al.*, 1991; Carter *et al.*, 1983).

I.2.3 Maintenance of Viral Persistence in the CNS

I.2.3.1 Mechanisms of Persistence

It is thought that measles virus entering the CNS is not defective, and that the initial stage of infection in the CNS involves an acute infection producing cell free virus, since an M gene sequence isolated from the brain tissue of an SSPE patient resembled an acute disease measles virus strain (Baczko *et al.*, 1993). Persistent infection of lymphocytes or monocytes has been suggested for the pathogenesis of measles virus associated disease processes including CNS complications (Cosby *et al.*, 1989; Cosby, 1993). The establishment of persistence may occur due to down-regulation of transcription. Thus, the integrity of the viral protein functions associated with transcription and intracellular

replication may be maintained, while the expression of the envelope glycoproteins is attenuated or abolished. The mechanism of measles virus persistence is thought to be host controlled as the transcription initiation sequence and viral polymerase encoding gene differ only slightly in persistent measles virus when compared to acute measles virus. At a later stage of infection only cell-associated virus can be detected. Carrigan (1985; 1986) has described two distinct variants of the same virus associated with the lytic and persistent stages of infection. A round cell (RC) variant of measles virus is found during the first stage of infection, producing cell free virus; the second variant is called the syncytiagenic (S) form. This variant does not release virus from the cell and little or no expression of viral M protein is observed. This form is associated with neurovirulence (Carrigan, 1985). The RC variant shows little or no neurovirulence, and there was a lack of histopathological changes in infected CNS tissues during the period when production of the RC variant was the greatest. No acute or histopathological disease occurred in newborn hamsters infected intra-cerebrally (i.c) with dilutions of the virus stock containing only the RC variant. The RC variant is capable of converting to the syncytiagenic form but the stimulus for the conversion is unknown, although the mechanism is thought to be by mutation. Conversion to the syncytiagenic form correlates with an increase in antiviral immune responses, therefore not only the state of infection, but also the selective decrease of the RC variant with respect to the syncytiagenic variant are due to immune responses to infection (Carrigan, 1986). Inter-conversion between these two variants may provide a mechanism for change from productive infection to defective infection of measles virus in the CNS.

In the later stages of infection measles virus is able to maintain a persistent infection by attenuating the expression of M, F and H proteins due to low mRNA frequencies and / or

the incorporation of mutations in the reading frame of those genes leading to defective expression or the abolishment of expression. Reduced expression of the membrane glycoproteins results in low expression of the major viral antigenic determinants on the cell surface, thus there is reduced recognition of infected cells by the host immune system. Down-regulation of measles virus gene expression in human brain cells can occur by a cell type-dependent regulation of viral mRNA transcription and a differentiation-dependent regulation of translation, both of which may be important for establishment of persistent measles virus infection in the CNS (Schneider-Schaulies *et al.*, 1993).

The Biken strain of SSPE dominantly interferes with the replication of wild-type measles virus in cultured cells. The mechanism of such interference is unknown, however the ability of a mutant virus to interfere with the growth of wild type virus would confer a selective advantage to the mutant virus in a long-term mixed infection, as is seen in the CNS during SSPE. Such a mutant virus could eventually predominate and displace the parental virus (Hirano, 1992a).

I.2.3.2 Role of Host Regulatory Factors in the Maintenance of Persistence

Host regulatory factors must also play a role in the maintenance of a persistent measles virus infection. Specific binding of various, uncharacterised, host proteins to the leader sequence of measles virus has been shown in many cell lines, including brain cells (Leopardi *et al.*, 1993). Total cellular phosphorylation in persistent measles virus infections of most neuroblastoma cell lines has been shown to increase by 50%, which correlates with an increase in protein kinase C activity. The release of infectious measles

virus was strongly inhibited by incubating the infected cells with a protein kinase C inhibitor, so suggesting that measles virus induces phosphorylation at a cellular level, which is essential for measles virus production. It was shown that the proteins phosphorylated were of cellular origin and that their phosphorylation was induced by the virus. These results suggest that the enhanced phosphorylation activity of persistently infected cells is induced by the virus and that protein kinase C-mediated phosphorylation plays an important role in the maturation of infectious measles virus (Segev *et al.*, 1994).

Increased protein tyrosine kinase activity has been seen in cells persistently infected with measles virus. The NP protein of measles virus in such cells is phosphorylated on tyrosine in addition to the normally phosphorylated serine and threonine residues in acute infections. Phosphorylation of the tyrosine residue on NP protein may introduce conformational changes which interfere with the interaction of NP with the M protein so preventing the assembly and release of virus progeny (Segev *et al.*, 1995).

As already described (I.2.2.2) DRADA may promote biased hypermutation which may contribute to the restriction of viral replication. This enzyme is thought to be a cellular house-keeping enzyme important in editing cellular RNA and destabilising duplex RNA to facilitated their degradation by RNAses.

The induction of cytokines may also help to maintain the persistent infections. Type I IFN (α and β) is induced in brain cells during persistent measles virus infections. Recent studies suggested that interferon induces MxA protein which can interfere with measles

virus replication in a tissue specific manner (Schneider-Schaulies *et al.*, 1994a). Inhibition of measles virus replication at the level of primary RNA synthesis only occurs when MxA is expressed in brain cells.

Persistent infections of C6 rat astrocytoma cells with SSPE or canine distemper virus (also associated with neurological disease in dogs) caused the loss of endothelin-1 (ET-1) binding to its receptor and, subsequently, the loss of the ET-1 induced Ca^{2+} signalling pathway. Endothelins are vasoconstrictive peptide factors, whose receptors are found in the CNS, endothelial cell and fibroblasts among others. Down-regulation of the ET-1 receptor might be advantageous for the establishment of persistent infections because cells are then less responsive to exogenous ET-1 stimuli, resulting in reduced phosphatidylinositol biphosphate (PIP_2) cleavage and the loss of Ca^{2+} signalling. However, PIP_2 cleavage has been shown to stimulate measles virus replication (Vainionpaa *et al.*, 1991; Meissner and Koschel, 1995).

I.2.3.3 Role of Defective Interfering Particles in Persistent Infections

Brain cells of patients with SSPE have been shown to contain mutant measles virus genomes with 5' copy-back defective interfering (DI) RNA's (Sidhu *et al.*, 1994). The role of measles virus DIs in persistent infection has been suspected for some time (Rima *et al.*, 1977). How DIs contribute to the development and survival of persistent infection of brain cells and the restriction of measles virus gene expression is still unknown. The following theory has been suggested to account for the presence of the DI particles and their possible role. SSPE infected brains may lack the high degree of selective pressure

encountered in tissue culture measles virus infections, thus, allowing the co-existence of numerous replication-competent defective particles. Such defective RNAs may act to limit full length measles virus genome replication by providing a pool of measles virus replicative promoter sequences which effectively competes for replicative polymerase complexes. As such, they are likely to reduce the measles virus transcriptional efficiency by limiting the abundance of polymerase complexes available to engage the promoter and initiate transcription, thus, explaining the low levels of mRNA detected in SSPE virus infected cells (Sidhu *et al.*, 1994).

I.2.3.4 Summary

The persistence of measles virus in the CNS is likely to be the result of both host regulatory factors and mutational mechanisms that significantly alter the genome of the virus. Such mutated virus could then be selected for propagation in the CNS. An effective way of maintaining a persistent infection would be to attenuate the expression of the membrane-associated proteins i.e., F, H and particularly M, as the M protein is responsible for facilitating viral assembly and budding. As previously explained, the M protein of SSPE viruses have been shown to contain many U-C transitions (Cattaneo *et al.*, 1986; Wong *et al.*, 1989). Propagation of the Yamagata-1 SSPE virus in neuroblastoma cells has been shown to induce additional U-C transitions (Wong *et al.*, 1989), leading to the hypothesis that a cellular factor may be responsible for the mutational activity. The theory that a nuclear located enzyme (DRADA) (Bass *et al.*, 1989) is responsible for such biased hypermutations may be mis-leading as measles virus does not require the cell nucleus for replication. It, therefore, remains unclear as to

whether the biased hypermutations seen in SSPE viruses, which may have extreme consequences for the virus, occur as the result of growth in neural cells or as a function of the viral genome itself. It is one of the aims of this project to analyse this process in greater detail.

I.CHAPTER 3

GENETIC HETEROGENEITY OF MEASLES VIRUS

I.3.1 Antigenic Variation

Measles virus has been considered as monotypic as only one serotype has been identified. Monoclonal antibody studies have demonstrated, however, that a number of strains differ in their patterns of antibody binding (Sheshberadaran *et al.*, 1983). M and H protein appeared to have the strongest degree of variation between strains while P, NP and F proteins were antigenically more stable. Giraudon *et al.* (1988) confirmed the presence of epitopic variation in measles virus when studying the antibody binding affinities of the NP protein of different strains of measles virus.

I.3.2 Genetic Variation

Comparisons and alignments of paramyxovirus and morbillivirus major structural protein sequences (Rima, 1989) have shown that the carboxy-terminal part of the NP and the amino-terminal 100 amino acids of P and C (Baczko *et al.*, 1992) are variable. The H protein is slightly less variable than the NP and P.

The H and NP protein coding sequences have been extensively analysed because these proteins elicit strong immune responses in the host. The carboxy-terminal 151 amino acids of the NP protein were originally analysed in 46 strains of measles virus. These strains were found to have up to 7.2% divergence at the nucleotide sequence level and 10.6% divergence at the amino acid sequence level (Taylor *et al.*, 1991a). The measles virus strains were found to fall into 6 different genotypic groups, some of which have not

been isolated recently, and so are regarded as extinct. Analysis of the H protein nucleotide sequence confirmed the division of the measles virus strains into the various lineage groups (Rima *et al.*, 1995a) previously described by Taylor *et al.* (1991a). Variation in the H gene may be an expression of the immunological pressure on the protein as it is the main immunogen for neutralising antibodies. However, it is also expected that the necessity of the H protein to bind to the CD46 membrane receptor places a greater structural constraint on the protein. More recent data utilising the nucleotide sequence of the carboxy-terminal region of the N protein in 65 measles virus strains, including viral RNA isolated from SSPE patient tissue, has identified a possible 8 different genotypes (Rima *et al.*, 1995b) (Table 1). The different genotypes are not geographically restricted and there appear to be at least 4 co-circulating genotypes at present.

Group A contains all vaccine strains used to date, and is typified by the Edmonston strain of measles virus. This group also contains early American isolates (Rota *et al.*, 1994a) and three SSPE derived strains, Halle, Mantooth and Horta-Barbosa (Horta-Barbosa *et al.*, 1971). Group A is homologous with the group 1 genotypes described by Taylor *et al.* (1991a).

Group B contains two clusters of African isolates from two independent epidemics in Gabon (1984) and Cameroon (1983). The isolates are divergent among themselves, and so are grouped separately, B1 corresponds to the Cameroon isolates and B2 to those from Gabon (Taylor *et al.*, 1991a; Rota *et al.*, 1994b). This group is homologous with the group 6 genotype described by Taylor *et al.*, 1991a). Measles virus isolates from the

Gambia were found to form a distinct group when the H gene sequences were compared to other African isolates, confirming that African isolates are divergent among themselves (personal communication, Dr. M. Outlaw, University of Warwick).

Group C is divided into 3 sub-groups. C1 group is typified by the SSPE isolate MF, and contains mostly SSPE isolates with the exception of two wild-type isolates from Madrid in 1978 and 1979. This sub-group may represent an extinct genotype as the last representatives were identified in the late 1980's. C1 is homologous with group 2 identified by Taylor *et al.* (1991a). The C2 group is typified by strain JM isolated from the USA in 1970. This genotype has been reported in the 1980s from Germany and Austria, and since 1990, every year in Germany, in Holland in 1991 and in 1992/93 in Spain. This group circulates currently in populations with high vaccination rates, and is homologous with group 4 genotype described by Taylor *et al.* (1991a). The C3 group contains only one virus isolate, SBI (Bonn, Germany). This virus probably represents a novel imported virus genotype rather than drastically mutated C group viruses.

Group D also contains 3 sub-groups: D1 contains 4 isolates related to the MVO strain (Bristol, 1974). The viruses in sub-group D1 are homologous with those in group 5 as described by Taylor *et al* (1991a). The first isolates in the sub-group D2 were from the UK in 1988 and this strain was still circulating in the UK in 1991. A related strain was isolated from Canada in 1989 and caused a major outbreak in the USA in 1989/90 (Rota *et al*, 1994b).

The earliest isolate in group E is the Woodfolk strain. It does not appear to be circulating at present, and may, therefore, be extinct. This group is homologous with group 3 virus strains described by Taylor *et al* (1991a).

Group F contains only two SSPE isolates isolated in 1979 and 1994 from two patients who had an acute measles virus infection in 1967 and 1968, respectively. The wild-type virus circulating at the time of onset of the symptoms of SSPE belongs to group C, which circulated from 1970. However, the virus isolated from the patients is not of group C lineage. This evidence supports the hypothesis that the wild-type virus circulating at the onset of SSPE symptoms is not the strain that gives rise to the persistent infection. It is more likely that the persistent virus isolated from these patients belongs to a virus lineage which was circulating in 1967/8 at the time of their primary infection.

Group G contains two wild-type isolates from the USA in 1983 (Rota *et al*, 1994b). They are distinct from any of the other groups and may represent imports from a group circulating in other countries or an extinct lineage group.

This information indicates that virus genotypes belonging to the sub-groups C2 and D2 may be still circulating, and that genotypes belonging to the groups A, E and F have not been isolated recently and may be extinct.

All measles virus strains isolated before 1970, with the exception of two SSPE strains isolated in Madrid in 1968 and 1967, belong to group A, along with all the vaccination strains. Vaccination with Edmonston-derived vaccines was started, in many countries,

Table 1. Group relationships between several measles virus strains

Group	Name	Description	Sequence Reference
A	Edm V	Edmonston-Enders vaccine/USA/1954	Rozenblatt *et al.* (1985)
	Edm P9	SDA variant of Edm strain	Taylor *et al.* (1991)
	Edm B	Edm B vaccine	Rota *et al.* (1994a)
	Edm wt	Edm Wild-type/USA/1954	Rota *et al.* (1994a)
	Edm wtpf	Edm Wild-type/USA/1954 plaque pure	Rima *et al.* (1995)
	Edm Zag	Edmonston-Zargreb vaccine	Rota *et al.* (1994a)
	Schw	Schwarz vaccine	Rota *et al.* (1994a)
	Hu2	Schwarz-vac. related case/N. Ireland/1971	Taylor *et al.* (1991)
	Mor 1	Moraten vaccine	Rota *et al.* (1994a)
	Mor 2	Moraten-vac. related case/Netherlands/1990	Taylor *et al.* (1991)
	CAM	Vaccine from Tanabe strain/Japan/1968	Rota *et al.* (1994a)
	Len	Leningrad-16 strain/Russia/1960	Rota *et al.* (1994a)
	S191	Shanghai vaccine/China/1960	Rota *et al.* (1994a)
	Chg	Changchun-47 vaccine/China/1957	Rota *et al.* (1994a)
	AIK-C	Edm-derived vaccine	Mori *et al.* (1993)
	Hln	Halonen strain/Finland/1962	Rota *et al.* (1994a)
	Ph 26	Philadelphia-26 strain/USA/1957	Rota *et al.* (1994a)
	Hal	Halle SSPE isolate/USA/1971	Buckland *et al* (1988)
	Man	Mantooth SSPE strain/USA/1971	Rima *et al* (1995)
	HB	Horta-Barbosa SSPE strain/USA/1971	Rima *et al* (1995)
	*0077	Wild-type/Coventry UK/1993	Outlaw & Pringle (1995)
B1	Y22	Wild-type/Cameroon/1983	Taylor *et al* (1991)
	Y14	Wild-type/Cameroon/1983	Rota *et al* (1994b)
B2	R118	Wild-type/Gabon/1984	Taylor *et al* (1991)
	R103	Wild-type/Gabon/1984	Rota *et al* (1994b)
	R113	Wild-type/Gabon/1984	Rota *et al* (1994b)
	R96	Wild-type/Gabon/1984	Rota *et al* (1994b)
C1	MF	SSPE case/Europe/early 1970s	Rima *et al* (1995)
	SIP3A	SSPE case 1P3/USA/early 1970s	Cattaneo *et al* (1989b)
	S(A)	SSPE case A/Germany/mid 1980s	Cattaneo *et al* (1989b)
	YA	SSPE case Yamagata-1/Japan/late 1980s	Yoshikawa *et al* (1990)
	SMa81	SSPE case/Madrid/1981/1970	Rima *et al* (1995)
	S(K)	SSPE case K/Germany/mid 1980s	Rima *et al* (1995)
	Mad78	Wild-type/Madrid/1978	Rima *et al* (1995)
	Mad79	Wild-type/Madrid/1979	Rima *et al* (1995)
C2	JM	Wild-type/Bethesda USA/1977	Taylor *et al* (1991)
	S(B)	SSPE case B/Austria/mid 1980s	Cattaneo *et al* (1989b)
	WTF	Wild-type/Germany/1990	Rima *et al* (1995)
	Bil	Wild-type/Netherlands/1991	Rima *et al* (1995)
	DL	Wild-type/Germany/1992	Rima *et al* (1995)
	LB	Wild-type/Germany/1993	Rima *et al* (1995)
	Ma92A	Wild-type/Madrid/1992	Rima *et al* (1995)
	Ma92R	Wild-type/Madrid/1992	Rima *et al* (1995)
	Ma93F	Wild-type/Madrid/1993	Rima *et al* (1995)
C3	SBI	Wild-type/Bonn Germany/1992	Rima *et al* (1995)
D1	MVO	Wild-type/Bristol UK/1974	Taylor *et al* (1991)
	MVP	Wild-type/Bristol UK/1974	Taylor *et al* (1991)
	S33	SSPE/N.Ireland/1983	Taylor *et al* (1991)
	S81	SSPE/N.Ireland/1986	Taylor *et al* (1991)

Continuation of Table 1.

Group	Name	Description	Sequence Reference
D2	CL	Wild-type/Birmingham UK/1988	Schulz *et al* (1992)
	SE	Wild-type/Birmingham UK/1988	Schulz *et al* (1992)
	TT	Wild-type Kawasaki case/London/1991	Schulz *et al* (1992)
	Can	Wild-type/Canada/1989	Rota *et al* (1994b)
	Chil	Wild-type/Chicago USA/1989	Rota *et al* (1994b)
	Chi2	Wild-type/Chicago USA/1989	Rota *et al* (1994b)
	SD	Wild-type/San Diego USA/1989	Rota *et al* (1994b)
	JK	MIBE/USA/1990	Rota *et al* (1994b)
D3	Ma94B	Wild-type/Madrid/1994	Rima *et al* (1995)
E	Brx	Encephalitis case/Germany/1971	Rima *et al* (1995)
	WFK	Wild-type/ Woodfolk USA/early 1970s	Rima *et al* (1995)
	CM	Wild-type/USA/late 1970s	Taylor *et al* (1991)
	S(C)	MIBE/USA/late 1970s	Cattaneo *et al* (1989b)
F	SMa79	SSPE case/Madrid/1979/1967	Rima *et al* (1995)
	SMa94	SSPE case/Madrid/1994/1968	Rima *et al* (1995)
G	Be83	Wild-type/Berkeley USA/1983	Rota *et al* (1994b)
	B083	Wild-type/Boston USA/1983	Rota *et al* (1994b)

Adapted from Rima *et al*, 1995a

* Determined from sequencing the H gene. Four other 1993 Coventry isolates (041, 049, 081 & 105) were found to belong to a unique lineage when compared with other measles virus strains whose lineages were determined by sequencing the H gene. As this table represents measles virus lineages with respect to the N gene it is not appropriate to incorporate the Coventry isolates into the table.

after 1971, thus it seems unlikely that immunisation programmes have been responsible for the disappearance of the Edmonston genotype and its replacement by other genotypes. Some of the other genotypes appear to have been circulating for at least 15 years, suggesting that they represent long established genotypes.

There is no indication that currently used vaccines can not control measles virus infection with genotypes different from that of the Edmonston strain. Antibodies to the vaccine strain of measles virus can neutralise wild-type virus of a different genotype, suggesting that one or several neutralising epitopes are conserved between vaccine and circulating wild-type strains. However, differential reactivity patterns of serum from infected persons suggest that circulating wild-type virus contains new or modified epitopes that are not present on the vaccine strain (Tamin *et al*, 1994; Rima *et al*, 1995b). It is not clear

how this may affect the ability of new wild-type viruses to replicate and be transmitted to persons with waning titres of immunity generated by vaccination with a different genotype; or what effect it may have on the infection rates in children with waning maternal antibody responses generated by vaccination with Edmonston-strain or by infection with wild-type viruses of a different genotype.

Very few silent changes are found in comparisons of similar strains, and the number of expressed mutation between genotypes is large. This indicates that selective forces are operating on the virus, which are not expected to favour silent mutations. To what extent the genetic variations observed between the different strains is driven by the immune system remains to be determined. It is also impossible to determine which, if any mutations observed, are associated with adaptation of the virus to propagation *in-vitro*.

The groupings described are consistent and independent of the area of the genome compared. Comparisons of the H, P, M and N genes show the same pattern of genotypes (Taylor *et al.*, 1991a; Baczko *et al.*, 1991, 1992; Rota *et al.*, 1992, 1994a, b; Outlaw and Pringle, 1995). This indicates the absence of recombination between the genotypes.

I.4.0 Aims

The overall aim of this project was to establish a system whereby the phenomenon of biased hypermutation could be reproduced *in-vitro*, confirming the observation of Wong *et al.* (1989), and to determine whether biased hypermutation could be induced in acute disease or vaccine-related measles virus, or in other non-segmented genome RNA viruses not normally found in neural tissue. If biased hypermutation could not be induced *in-vitro* in acute disease measles virus, the aim was to establish whether this phenomenon is a property specific to SSPE viruses.

Human respiratory syncytial virus was chosen for analysis in this project because it belongs to the *Paramyxoviridae* family of viruses, along with measles virus, and it does not involve the CNS during infection. DRADA, whose action has been suggested to play a role in the production of hypermutation events, is ubiquitous. It therefore, seems unlikely that this enzyme would only target measles virus associated with the CNS. If biased hypermutation can be reproduced *in-vitro* within the genome of respiratory syncytial virus, where there is no nuclear involvement in its replicative cycle, this would be good evidence of cellular influence on the viral genome, rather than a nuclear-associated effect.

Comparisons of the frequency of biased hypermutation events in virus material propagated in cells of neural and non-neural origin would also be determined, as the DRADA activity has been shown to be higher in neuroblastoma cells when compared to Vero cells.

PART II
METHODS & MATERIALS

II.CHAPTER 1

METHODS

II.1.1 Cell Culture

II.1.1.1 Growth of African Green Monkey Kidney Cells (Vero)

African green monkey kidney cells (Vero), ATCC No. CCL 81, were cultured in 175 cm^2 plastic flasks at 37°C in Dulbecco's Modification of Eagle's media (DMEM) supplemented with L-glutamine (4 mM), penicillin (100 units ml^{-1}), streptomycin (100 μ g ml^{-1}) and 5% (v/v) foetal calf serum in an atmosphere of 5% CO_2.

Cells were passaged when confluent, typically at 3 day intervals, at a ratio of 1:5. Confluent cells were given two washes with 10 ml of versene, both for approximately 1 minute at room temperature. Cells were removed from the surface of the flask with 10 ml of a 0.25% (w/v) trypsin / versene solution to produce a cell suspension. Two ml of this suspension was introduce to a new flask containing 50-75 ml of supplemented DMEM.

II.1.1.2. Growth of Human Lung Fibroblast Cells (MRC-5)

Human lung fibroblast cells (MRC-5), ATCC No. CCL 171, were cultured in 175 cm^2 large plastic flasks at 37°C in Glasgow Modification of Eagle's media (GMEM) supplemented with L-glutamine (4 mM), penicillin (100 units ml^{-1}), streptomycin (100 μ g ml^{-1}) and 10% (v/v) foetal calf serum in an atmosphere of 5% CO_2.

Cells were passaged when confluent, typically at 4 to 5 day intervals, at a ratio of 1:3. Confluent cells were given two washes with 10 ml of phosphate buffered saline (PBS),

the first was for 1 minute at room temperature, the second for 10 minutes at 37°C. Cells were finally removed from the flask surface with 10 ml of a 0.25% (w/v) trypsin / PBS solution to produce a cell suspension. Approximately 3.3 ml of this suspension was introduced to a new flask containing 50-75 ml of supplemented GMEM.

II.1.1.3 Growth of Neuroblastoma Cell Line (IMR-32)

The neuroblastoma cells (IMR-32), ATCC No. CCL 127, were cultured in 175 cm^2 large flasks at 37°C in Dulbecco's Modification of Eagle's media (DMEM) supplemented with L-glutamine (4 mM), penicillin (100 units ml^{-1}), streptomycin (100 μg ml^{-1}) and 10% (v/v) foetal calf serum in an atmosphere of 5% CO_2.

Cells were passaged when confluent, typically at 2 to 3 day intervals, at a ratio of 1:2. Confluent cells were given two washes with 10 ml of PBS, both for 1 minute at room temperature. Cells were removed from the surface of the flask by the addition of 10 ml of PBS directly to the cell monolayer, producing a cell suspension. Five ml of this suspension was introduced to a new flask containing 50-75 ml of supplemented DMEM.

II.1.1.4 Growth of Neuroblastoma Cell Line (SK-N-SH)

The neuroblastoma cells (SK-N-SH), kindly provided by Dr. S. Swingler (University of Warwick), were cultured in 175 cm^2 plastic flasks at 37°C in Dulbecco's Modification of Eagle's media (DMEM) supplemented with L-glutamine (4 mM), penicillin (100 units ml^{-1}), streptomycin (100 μg ml^{-1}) and 10% (v/v) foetal calf serum in an atmosphere of 5% CO_2.

Cells were passaged when confluent, typically at 2 to 3 day intervals, at a ratio of 1:2. Confluent cells were given two washes with 10 ml of versene, both for 1 minute at room temperature. Cells were removed from the flask surface with 10 ml of a 0.25% (w/v) trypsin / versene solution to produce a cell suspension. Five ml of this suspension was introduced to a new flask containing 50-75 ml of supplemented DMEM.

II.1.2 Virus Propagation *In-Vitro*

II.1.2.1 Measles Virus Propagation *In-Vitro*

The Edmonston strain of measles virus obtained from the American Type Culture Collection (ATCC No. VR-24) was used to infect 4 cell lines; 2 neuroblastoma cell lines (IMR-32 and SK-N-SH), human diploid lung fibroblast cells (MRC-5) and monkey kidney epithelial cells (Vero). The virus had previously been passaged 24 times in human kidney cells, followed by 33 passes in human amniotic cells. For this experiment the virus was then passaged a further 10 times in each of the cell lines mentioned above.

After each passage the virus was harvested; 0.2 ml of the harvested virus, diluted 1:100, was used for further passage, the remainder was stored in 1 ml aliquots at -70°C until required. RNA was extracted (Kumar & Lindberg, 1972) and cDNA synthesised (II.3.1 & II.3.2) from passages 1 and 10 in preparation for the amplification of the M gene by polymerase chain reaction (II.3.3.1).

II.1.2.2 Human Respiratory Syncytial Virus Propagation *in-vitro*

A non-neurotropic paramyxovirus was required as a control for the passage experiments. Respiratory syncytial virus (HuRS virus) was provided by Dr. Patricia Cane (University of Warwick). The RSS-2 strain, previously passaged 7 times in MRC-5 cells only, was used. This virus was passaged in 3 cell lines; the two neuroblastoma cell lines (IMR-32 and SK-N-SH) and the human diploid lung fibroblast cells (MRC-5). As for measles virus, HuRS virus was passaged 10 times in each of the cell lines. The virus was harvested after each passage; 0.2 ml of the harvested virus was used for further passage, the remainder was stored in 1 ml aliquots at -70°C until required. RNA was extracted (Kumar & Lindberg, 1972) and cDNA synthesised (II.3.1 & 11.3.2) from passages 1 and 10 in preparation for the amplification of the M gene by polymerase chain reaction (II.3.3.1).

II.1.2.3 Propagation of Yamagata-1 Virus

Yamagata-1 virus (YM-1), kindly provided by Professor Homma (Yamagata University School of Medicine, Japan), is a measles virus isolated from the brain of a deceased SSPE patient by co-cultivation of the brain tissue with monkey kidney epithelial cells (Vero). Two passage strategies were employed for this experiment.

I: YM-1 virus was passaged directly in the 2 neuroblastoma cell lines (IMR-32 and SK-N-SH). The virus was passaged up to 5 times in each cell line. One Yamagata-1 virus infected flask (25 cm^2) was split 1:2 into 25 cm^2 flasks containing freshly dispersed cells. One flask was used to harvest total RNA by freeze/thawing and aliquoting the cells into 1 ml portions; the infected cell monolayer from the remaining flask was harvested by treatment with EDTA/trypsin (GIBCO BRL) and split 1:2 for further passage experiments. The IMR-32 cells did not need treatment with the EDTA/trypsin in order to disperse the cells, gentle rocking of the monolayer sufficed. RNA was extracted (Kumar & Lindberg, 1972) from the frozen aliquots and cDNA synthesised (II.3.1 & II.3.2) from passages 1 and 5 in preparation for the amplification of the M gene by polymerase chain reaction (II.3.3.2).

II: This strategy follows the procedure described by Wong *et al* (1989), who passaged the YM-1 virus in embryonic lung cells prior to passage in the neuroblastoma cell line (IMR-32). To determine if passage in the lung cells was important in the development of mutations in the virus genome, the

Yamagata-1 virus was initially passaged 3 times in human lung fibroblast cells (MRC-5). Virus harvested from passage 3 (as for strategy I) in the MRC-5 cells was then used as the inoculum for passage 1 in each of the 2 neuroblastoma cell lines (IMR-32 and SK-N-SH). The method of virus passaged was as described for strategy I. RNA was extracted (Kumar & Lindberg, 1972) from the frozen virus aliquots and cDNA synthesised (II.3.1 & II.3.2) in preparation for amplification of the M gene by polymerase chain reaction (II.3.3.2).

II.1.2.4 Plaque Assays

All plaque assays were carried out using Monkey kidney epithelial cells (Vero). Cells were grown on 6 well plates. The virus was diluted 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5} and 10^{-6}. The medium was removed from each well, leaving approximately 250 µl which should just cover the cell monolayer. Each well was inoculated with 250 µl of either medium, for the mock infection, or virus. The virus dilutions were inoculated in duplicate, from which the standard error could be calculated. Virus was left to adsorb for up to 1 hour at 33°C. The inoculum was removed by aspiration and 5 ml of agar overlay (100 ml 2% (w/v) agar, 100 ml 2x DMEM [4% foetal calf serum, 8 mM glutamine, 200 µg/ml streptomycin and 200 units/ml penicillin]) was added to each well. The infections were incubated for 7 days at 33°C. The cells were fixed by adding 2 ml glutaraldehyde (2% v/v) to each well and incubating at room temperature for at least 4 hours. The agar overlay was removed and the cells were stained using crystal violet (1.5% w/v crystal violet in ethanol, diluted 1:20 with water). The plaques could then be counted under the microscope.

II.1.3 Amplification of Genetic Material

II.1.3.1 Extraction of RNA from Infected Cells

The method of RNA extraction was derived from Kumar & Lindberg, (1972). A 1 ml aliquot of passaged virus was centrifuged for 3 mins. at 13,000 rpm in order to pellet the cells. The cells were then resuspended in 0.25 ml of ice cold isotonic lysis buffer (150 mM NaCl, 1.5 m M $MgCl_2$, 10 mM Tris pH 7.8, 0.65 % Nonidet P40), 0.25 ml of phenol extraction buffer (7.0 M Urea, 350 mM NaCl, 10 mM EDTA, 1 % SDS, 10 mM Tris pH 7.8) and 0.5 ml of equilibrated phenol / chloroform (1:1). The resulting mix was vortexed and centrifuged for 10 mins. at 13,000 rpm. The upper aqueous layer was removed and re-extracted using 0.5 ml of phenol / chloroform (1:1). The upper aqueous layer was retained and 2 times its volume of absolute ethanol was added. This was stored at -20 ^{o}C until required.

II.1.3.2 Production of cDNA

For each cDNA reaction the RNA extracted from a 1 ml aliquot of infected cells was centrifuged for 20 mins. at 13,000 rpm. The pelleted RNA was then washed in 0.5 ml 70 % ethanol and dried under vacuum. The pellet was then resuspended in 0.5 mM dATP, 0.5 mM dCTP, 0.5 mM dGTP, 0.5 mM dTTP, 50 mM Tris-HCL (pH 8.3), 40 mM KCl, 1 mM DTT, 6 mM $MgCl_2$, 4 μg oligo T primer, and 200U Superscript II RNase H^- reverse transcriptase in a final volume of 50 μl. The reactions were incubated at 37^{o}C for 1 hr, and stored at -20^{o}C.

II.1.3.3 Polymerase Chain Reaction (PCR)

II.1.3.3.1 Measles Virus and Respiratory Syncytial Virus M Gene Amplification

Each PCR reaction was carried out using 0.5 mM of each of the following, dATP, dCTP, dGTP and dTTP, 6 µl of cDNA, 0.4 µg of primer, 0.4 mM DTT, 4 mM $MgCl_2$, 30 mM KCL, 50 mM Tris.HCl (pH 8.3) and 2.5 units of *Thermus aquaticus* (Taq) DNA polymerase in a final volume of 100 µl. The reactions were overlaid with 30 µl of paraffin oil to prevent evaporation. Measles virus M gene PCR using the MMO1/MMO2 primer pair were thermally cycled at 94°C for 45 secs, 58°C for 45 secs and 74°C for 2 mins. for a total of 30 cycles; for measles virus M gene amplification using either MNO1/MMO2 or MVM1/oligo T, the cycles used were 94°C for 45 secs, 54°C for 45 secs and 74°C for 2 mins. (30 cycles). For amplification of the M gene of HuRS virus the cycles used were 94°C for 45 secs, 54°C for 45 secs and 74°C for 45 secs for a total of 30 cycles.

II.1.3.3.2 SSPE (Yamagata-1) virus M Gene Amplification

Each PCR reaction was carried out using 0.5 mM of each of the following, dATP, dCTP, dGTP, dTTP, 3 µl of cDNA, 10 mM KCl, 10 mM $(NH_4)_2SO_4$, 20 mM Tris-HCL (pH 8.0), 2 mM $MgSO_4$, 0.2 µg of primer, 0.1% Triton X-100 and 2 units of *Thermococcus litoralis* (Vent) DNA polymerase in a final volume of 50 µl. The reactions were overlaid with 20 µl of paraffin oil to prevent evaporation and thermally cycled at 94°C for 45 secs, 60°C for 45 secs and 74°C for 2 mins. for a total of 30 cycles.

II.1.3.3.3 SSPE (Yamagata-1) virus M Gene Amplification using Nested PCR

The first PCR reaction (reaction A) was carried out using 0.5 mM of each of the following, dATP, dCTP, dGTP, dTTP, 3 µl of cDNA, 0.2 µg of primer, 0.4 mM DTT, 4 mM $MgCl_2$, 30 mM Tris.HCl (pH 8.3) and 2.5 units *Thermus aquaticus*(Taq) DNA polymerase in final volume of 50 µl. The reactions were overlaid with 20 µl of paraffin oil to prevent evaporation and thermally cycled at 94°C for 45 secs, 56°C for 45 secs and 74°C for 1 min. for a total of 30 cycles.

The second PCR reaction (reaction B) was carried out using 0.5 mM of each of the following, dATP, dCTP, dGTP, dTTP, 3 µl of PCR reaction A, 0.2 µg of primer, 0.4 mM DTT, 4 mM $MgCl_2$, 30 mM Tris.HCl (pH 8.3) and 2.5 units *Thermus aquaticus* (Taq) DNA polymerase in final volume of 50 µl. The reactions were overlaid with 20 µl of paraffin oil to prevent evaporation and thermally cycled at 94°C for 45 secs, 56°C for 45 secs and 74°C for 1 min. for a total of 30 cycles.

II.1.4 Electrophoresis of DNA

II.1.4.1 Agarose Gel Electrophoresis

DNA fragments were run on agarose gels in order to analyse the products of PCR reactions, restriction digests and to separate fragments for purification. If fragments were to be purified from the gel a TAE buffer (40 mM Tris-acetate (pH 7.0), 1 mM EDTA, pH 8.3) was used, otherwise TBE buffer (100 mM Tris-base, 100 mM boric acid, 160 mM EDTA, pH 8.3) was the buffer of choice. To make the gel, agarose at 1% (w/v) was dissolved in near boiling buffer, cooled and poured into gel casting trays with well-forming combs. Ethidium bromide was added to the gel and the running buffer at 0.5 μ g/ml. DNA samples were supplemented with 20% (v/v) loading buffer (40% (w/v) glycerol, 7.5% (w/v) bromophenol blue, 10% (v/v) water) and pipetted into the sample wells. The DNA was electrophoresed at 150 mA until the bromophenol blue had travelled two thirds of the way down the gel. To determine the size of the electrophoresed DNA fragment, a 1 kb marker ladder was run alongside the samples. The DNA was visualised under ultraviolet light (365 nm) and photographed using Polaroid 667 instant black and white film.

II.1.4.2 Polyacrylamide Gel Electrophoresis

The products of sequencing reactions were separated by electrophoresis on polyacrylamide gels. To make the gel 5.7% (w/v) acrylamide, 0.3% (w/v) N,N'-methylene-bisacrylamide and 8 M urea were dissolved in 120 ml of 1 x TBE which was

filtered through No. 1 Whatman filter paper before pouring. Polymerisation of the gel was facilitated by the addition of 0.05% (v/v) N,N,N',N'-tetramethylethylenediamine (TEMED) and 0.5% ammonium persulphate. The gel was poured between two glass plates to form a wedge gel 0.4 mm thick at the top and 1.0 mm thick at the bottom. Short tooth combs were inserted at the top of the gel to form a straight gel front. The gel was left to polymerise before running. Before samples were loaded, the gel was warmed by pre-running at 80 W for 20 min., the samples were loaded on the gel (2.5 µl/well) and electrophoresed at 80 W for 1 hr 30 mins. to 8 hrs. The gel was fixed for 40 mins. in 10% acetic acid, dried on a heated vacuum drier and autoradiographed using Fuji X-ray film.

II.1.5 *In-Vitro* Manipulation of DNA

II.1.5.1 Purification of DNA Fragments from Agarose Gels

The PCR products were run out on agarose gels buffered with 1x TAE (40 mM Tris-Acetate, 1 mM EDTA, pH 8.3). The DNA fragments to be cloned were excised form the gel and purified using the Geneclean kit from BIO 101 (United States Biochemicals). This involved the addition of 3 volumes of 6 M sodium iodide and incubation at 55°C for 5 mins. or until the agarose dissolved. Five microlitres of a binding matrix (a silica suspension in water) was added, and mixed well before incubating for 5 mins. on ice. The binding matrix and DNA was pelleted at 13,000 rpm for 10 secs. and the supernatant discarded. The pellet was then washed 3 times with 300 µl of New Wash (NaCl/ethanol/water solution buffered with a mixture of Tris-acid and Tris-base to give a

pH of between 7.0 and 8.5. Information of precise mixture of Tris-acid and Tris-base not given by the manufacturer). In between each wash the resuspended matrix bound DNA was pelleted at 13,000 rpm for 5 secs, the supernatant was discarded and fresh wash solution added. After the final wash the pellet was centrifuged again for 5 secs. at 13,000 rpm in order to remove any residual supernatant. The DNA was then eluted by resuspending the matrix bound DNA in 10 µl of water and incubating for 3.5 minutes at 55°C. The mixture was then centrifuged at 13,000 rpm for 30 secs., the eluted DNA was transferred to a fresh eppendorf tube and the binding matrix was once more resuspended in 10 µl of water in order to elute the remaining DNA.

II.1.5.2 Restriction Enzyme Digestion of DNA

Double-stranded DNA was cleaved with a variety of restriction enzymes from a number of commercial suppliers. Typically a restriction digest would consist of 4 µl of DNA from a 100 µl PCR reaction or from a small scale DNA preparation, the appropriate buffer as recommended by the manufacturer and the restriction enzyme(s) as required. The restriction digests were incubated for 1 hr at a temperature recommended by the suppliers.

II.1.5.3 Ligations

Ligations were carried out in a small volume, typically 20 µl. at 16°C overnight. The reaction mix contained the appropriately digested vector (M13 mp18 or mp19), T4 DNA ligase buffer (50 mM Tris.HCl (pH 7.6), 10 mM $MgCl_2$, 1 mM ATP, 1 mM DTT, 5% (w/v) polyethylene glycol-8000), gel purified digested DNA fragment and 1 unit of T4 DNA ligase.

II.1.5.4 Cloning into M13

M13 mp18 or mp19 was digested with the appropriate enzymes (II.5.2) run on TAE agarose gels and purified using Geneclean (BIO 101). The purified DNA fragments were then ligated into the multiple cloning site of the M13 cloning vector prior to transformation into competent bacterial cells.

II.1.5.5 Cloning into TA Vector

Nested PCR products were gel purified and ligated directly into a TA vector (pGEMT from Promega) using T4 DNA ligase (II.5.3), prior to transformation into competent bacterial cells.

II.1.6 Transformation of Bacterial Cells with Cloned DNA

II.1.6.1 Bacterial Strain

Escherichia coli K12 strain TG2 was used for all transformations. The genotype of TG2 is supE hsd$^{\Delta}$5 thi$^{\Delta}$(lac-proAB)$^{\Delta}$(srl-recA)306::Tn10(tet^{r})F'[traD36 proAB^{+}lacI^{q} lacZ$^{\Delta}$ M15]. *E. coli* strain TG2 was maintained on L-broth plate (1% (w/v) NaCl, 1% (w/v) bactotryptone, 0.5% (w/v) yeast extract, 1.5% (w/v) bactoagar). Overnight cultures of *E. coli* strain TG2 were prepared by inoculating 50 ml of L-broth (0.5% (w/v) NaCl, 1% (w/v) bactotryptone, 0.5% (w/v) yeast extract) with a single colony, and shaking at 200 rpm at 37^{o}C overnight in an orbital shaker.

II.1.6.2 Preparation of Competent Bacterial Cells

One ml of TG2 overnight culture was inoculated into 50 ml of L-broth and shaken at 200 rpm, at 37^{o}C until the culture gave an optical density reading of 0.5 at 600 nm. The culture was then cooled on ice for 30 min. and the cells pelleted at 3000 rpm for 10 min., at 4^{o}C. The pellet was resuspended in 5 ml of ice cold 0.1 M $MgCl_2$ and pelleted again at 3000 rpm for 5 min., at 4^{o}C. The supernatant was discarded and the pellet resuspended in 2 ml of ice cold 0.1 M $CaCl_2$. The cell suspension was left on ice for a minimum of 30 min. prior to transformation.

II.1.6.3 Transformation of Bacteria with the Bacteriophage M13

One hundred microlitres of competent TG2 cells were put into Falcon tubes and mixed gently with DNA from ligation reactions (1 to 10 µl). This was incubated on ice for 30 min., then heat shocked at 42°C for 2 min. before being returned to the ice for a further 30 min. Three ml of 42°C top agar (0.6% (w/v) bactoagar, 1.6% (w/v) bactotryptone, 1% (w/v) yeast extract, 0.5% (w/v) NaCl), 100 µl of early log phase TG2 cells, 15µl 2.4% (w/v) IPTG and 30 µl 3% (w/v) X-gal were added to the DNA competent cells. After gentle mixing this was poured onto L-broth plates and allowed to set before incubating overnight at 37°C. Clear plaques were observed within the lawn of TG2 cells. Isolated plaques were picked with a sterile tooth-pick and inoculated into 3 ml of 2YT containing 90 µl of a TG2 overnight culture.

II.1.6.4 Transformation of Bacteria with the TA Vector pGEMT

Two hundred microlitres of competent TG2 cells were put into Falcon tubes and mixed gently with 10 µl DNA from ligation reactions. This was incubated on ice for 30 mins., then heat shocked at 42 °C for 2 mins. before being returned to the ice for a further 30 mins. Two hundred microlitres of L-Broth was added giving a final volume of 410 µl. A range of volumes of the transformed cells was spread onto AIX plates (1 % tryptone, 0.5 % yeast extract, 0.5 % sodium chloride, 1.5 % bactoagar, 100 µg/ml ampicillin, 20 µg/ml IPTG, 0.5 ml X-Gal [2%]), and incubated overnight at 37°C.

II.1.7 Preparation of Cloned DNA

II.1.7.1 Preparation of Double Stranded DNA

Single clear plaques were stabbed with sterile tooth picks and transferred to 3 ml of 2YT containing 90 µl of a TG2 overnight culture. Cultures were shaken at 200 rpm at 37°C for 5 to 6 hrs. The culture was poured into sterile eppendorf tubes and centrifuged at 13,000 rpm for 3 min. The supernatant was transferred to a fresh eppendorf tube containing 250 µl of 2.5 M NaCl, 20% (w/v) PEG6000 solution, gently mixed and incubated overnight at 4°C prior to single-stranded DNA preparation (II.7.3).

The pelleted double-stranded DNA was resuspended in 100 µl of resuspension buffer (50 mM glucose, 10 mM EDTA, 25 mM Tris.HCl [pH 8.0]) and 250 µl of lysis buffer (0.2 M NaOH, 1% SDS). The solution was mixed by inverting the tube several times and incubated on ice for 5 min. One hundred and fifty microlitres of ice cold neutralisation buffer (3 M KAc [pH 4.8]) was then added and mixed thoroughly by vortexing for 10 sec. Nuclear DNA and cell debris was removed by centrifugation at 13,000 rpm for 5 min. The supernatant was transferred to a fresh eppendorf containing 250 µl of phenol / chloroform (1:1), this mixture was vortexed then centrifuged for 5 min. at 13,000 rpm. The upper aqueous layer was transferred to a fresh eppendorf tube and the DNA was precipitated by the addition of 1 ml of ethanol and a 2 min. incubation at room temperature. The DNA was pelleted by centrifugation at 13,000 rpm for 10 min. The supernatant was discarded and the pellet washed by the addition of 500 µl of 70% ethanol

and then dried under vacuum. The DNA was resuspended in 50 µl of sterile TE buffer (10 mM Tris.HCl (pH 8.0), 0.1 mM EDTA) and stored at -20°C.

Double-stranded DNA was digested (II.5.2) with either the restriction enzymes used for cloning or Pvu II in order to determine if the M13 plaque did contain the cloned M gene. Pvu II has sites approximately 100 base pairs either side of the multiple cloning site in M13. Therefore the size of a positive M gene clone would be approximately 300 bases larger than the expected size of the inserted gene.

II.1.7.2 Preparation of Double-Stranded DNA Silica Particles

II.1.7.2.1 Preparation of the Binding Matrix

Twenty five grams of diatomaceous earth was added to 500 ml of water and left to settle for approximately 3 hours. The supernatant was aspirated and water was added to a final volume of 100 ml. The binding matrix was resuspended and left to settle, giving a final concentration of 250 mg/ml. This was stored at room temperature. Prior to DNA preparation, 1 ml of the binding matrix was resuspended in 25 ml of matrix resuspension buffer (4M guanidine thiocyanate, 50 mM Tris. HCl [pH 7.0], 20 mM EDTA) and stored in the dark (Carter & Milton, 1993).

II.1.7.2.2 DNA Preparation

One millilitre of an over night culture of bacteria was centrifuged at 13,000 rpm for 2 mins. The pellet was resuspended in 200 µl of resuspension buffer (50 mM Tris. HCl [pH 7.5], 10 mM EDTA, 100 µg/ml RNase A). Two hundred microlitres of cell lysis buffer (0.2 M sodium hydroxide, 1 % SDS) was added, and the solution was mixed by inversion. A further 200 µl of neutralisation buffer (5M potassium acetate, 11.5 % acetic acid) was added, and the solution was mixed by inversion. The solution was centrifuged for 5 mins. at 13,000 rpm and the supernatant was transferred to a fresh eppendorf tube. One millilitre of binding matrix (250 mg/ml) was added to the supernatant. This was incubated for 1 min at room temperature. The binding matrix was pelleted by centrifugation at 13,000 rpm for 20 secs. The supernatant was discarded and the pellet was washed with 1 ml of 80 % isopropanol, followed by a wash in 1 ml of acetone. The pellet was then left to air dry for 5 mins. The binding matrix was then resuspended in 60 µl of water and the DNA was eluted by incubation at 65 ^{o}C for 5 mins. The binding matrix was pelleted at 13,000 rpm for 5 mins. and the supernatant was transferred to a fresh eppendorf tube (Carter & Milton, 1993).

II.1.7.3 Preparation of Single-Stranded DNA

Single stranded DNA was precipitated overnight at 4^{o}C with a PEG 6000/ NaCl solution (II.7.1). The DNA was then pelleted by centrifugation at 13,000 rpm for 10 min. The resulting pellet was then resuspended in 120 µl of TE buffer (10 mM Tris.HCl (pH 8.0), 0.1 mM EDTA) and extracted with 50 µl phenol, then 50 µl chloroform. The upper

aqueous layer was removed and the single stranded DNA precipitated by the addition of 10 µl NaAc and 250 µl of ethanol. This was mixed thoroughly and incubated for 15 min. at -70°C. The DNA was pelleted by centrifugation at 13,000 rpm, washed in 500 µl of 70% ethanol, vacuum dried and resuspended in 30 µl of TE buffer.

II.1.8 Nucleotide Sequencing Methods

II.1.8.1 Nucleotide Sequencing the PCR Product

II.1.8.1.1 Automated Sequencing

PCR products to be sequenced using the 373A DNA Sequencer (Applied Biosystems) were extracted from agarose gels using the Geneclean Kit (II.5.1). One microgram of DNA and 3.2 pmol of primer were made up to a final volume of 10.5 µl. This was then sent for automated sequence analysis by Mrs. L. Ward. Sequencing was carried out using the Taq DyeDeoxy^{TM} Terminator Cycle Sequencing Kit (Applied Biosystems) kit. The cycles used in these reactions were 96°C for 30 secs.; 50°C for 15 secs.; 60°C for 4 mins. for a total of 30 cycles. The dyes were extracted by passage down a Sephadex G50 column. The eluant was then ethanol precipitated. The resulting pellet was resuspended in 4 µl of loading buffer and loaded onto the gel. The gel was run for 12 hrs using fluorescence detection of the bases.

II.1.8.1.2 Cycle Sequencing

Chain termination cycle sequencing enhances the ability to sequence small amounts of template DNA. Sequencing reactions were essentially DNA synthesis reactions carried out in the presence of chain-terminating (dideoxy) nucleotides. The United States Biochemical Δ Taq cycle sequencing kit was used for this method of sequencing. The first step, the labelling step, was run under conditions where the extension of the primers was limited to just a few nucleotides by using only three of the dNTPs. The labelling reaction was made up of the following: 0.5 pmol/μl of primer, 2 μl of reaction buffer (260 mM Tris.HCl [pH 9.5], 65 mM $MgCl_2$), 0.05 pmol DNA, 1 μl dGTP cycle mix, 1 μl dCTP cycle mix, 0.5 μl ^{35}S-dATP (10 μCi/μl), 5 μl H_2O and 2 μl Δ Taq Version 2.0 DNA Polymerase (diluted 1:8). This was overlaid with 15 μl of paraffin oil and cycled at 95°C for 45 secs, 48°C for 30 secs and 74°C for 10 secs, for a total of 35 cycles (for measles virus); for huRS virus primers RSM1a, 3a, 6a, the cycle used was 95°C for 45 secs, 64°C for 30 secs and 74°C for 10 secs, for a total of 35 cycles; and form primers RSM4a and 5a the cycle used was 95°C for 45 secs, 68°C for 30 secs and 74°C for 10 secs, for a total of 35 cycles. Termination tubes were set up containing 4 μl of the respective termination mixes (15 μM each of dATP, dCTP, dGTP and dTTP along with either 22.5 μM ddGTP or 300 μM ddATP or 450 μM ddTTP or 150 μM ddCTP). A 3.5 μl aliquot of the labelling reaction was added to each termination tube, and overlaid with 10 μl of paraffin oil to prevent evaporation. The reactions were cycled at 95°C for 45 secs, 58°C for 45 secs and 70°C for 20 secs, for a total of 30 cycles for measles virus; for huRS virus the cycle used was 95°C for 45 secs, 70°C for 45 secs and 74°C for 120 secs, for a total of 30 cycles. The reactions were stopped by the addition of 4 μl of stop

solution (95% formamide, 20 mM EDTA, 0.05% bromophenol blue and 0.05% xylene cyanol FF.

II.1.8.2 Sequencing of Single-Stranded DNA

The sequencing method used was that of dideoxy chain termination (Sanger *et al*, 1977) and was performed with reagents from US Biochemicals Sequenase Kits. Single-stranded DNA was prepared as previously described (II.7.3). Primers were annealed in a 10 µl reaction volume containing 7 µl of single-stranded DNA, 60 mM Tris. HCl (pH 7.5), 20 mM $MgCl_2$, 50 mM NaCl and 1 µl of primer (0.4 mg/ml). This reaction was incubated at 65°C for 10 mins. and then allowed to cool to 45°C. The labelling step was carried out at room temperature for 3 mins. with the addition of 1 µl DTT (0.1 M), 2 µl of labelling mix (1.5 µM dGTP, 1.5 µM dCTP, 1.5 µM dTTP), 0.5 µl ^{35}S dATP (10 µCi/µl), 2 µl Sequenase (1:8 dilution) to the 10 µl template/primer reaction. Extension and termination was carried out for 3 mins. at 37°C by transferring 3.24 µl of the labelling reaction to each of 4 pre-warmed termination mixes (2.5 µl in each). The sequencing reaction were stopped by the addition of 4 µl of stop solution (95% formamide, 20 mM EDTA, 0.05% bromophenol blue and 0.05% xylene cyanol FF) to each termination reaction.

II.1.8.3 Sequencing of Double-Stranded DNA

Double stranded DNA was sequenced using the dideoxy chain termination (Sanger *et al*, 1977) and was performed with reagents form US Biochemicals Sequenase Kits. Double stranded DNA was prepared using the Magic Mini prep method previously described

(II.7.2). Eight microlitres of double-stranded DNA was incubated for 5 mins. at room temperature with 2 M sodium hydroxide. To this, 7 µl of primer (20 pmol/µl), 3 µl sodium acetate (2M [pH 4.5]) and 75 µl of ice cold ethanol was added. The solution was then incubated at -70°C for 15 mins. in order to precipitated the annealed primer. The DNA was pelleted by centrifugation at 13,000 rpm for 15 mins, and washed with 1 ml of 70 % ethanol. The resulting pellet was vacuum dried and resuspended in 8 µl water and 2 µl Sequenase reaction buffer. The labelling reaction was carried out at room temperature for 10 mins, with the addition of 1 µl DTT (0.1 M), 2 µl label mix (1.5 µM dGTP, 1.5 µ M dCTP, 1.5 µM dTTP), 0.5 µl ^{35}S dATP (10 µCi/µl) and 2 µl Sequenase (1:8 dilution) to the 10 µl template / primer reaction. Extension and termination was carried out for 30 mins. at 45°C by transferring 3.5 µl of the labelling reaction to each of 4 pre-warmed termination mixes (2.5 µl in each). The sequencing reactions were stopped by the addition of 4 µl of stop solution (95 % formamide, 20 mM EDTA, 0.05 % bromophenol blue and 0.05 % xylene cyanol FF) to each termination reaction.

II.CHAPTER 2

MATERIALS

II.2.1 Cells and Cell Culture

IMR-32	American Type Culture Collection (No. CCL 127)
MRC-5	American Type Culture Collection (No. CCL 171)
Vero	American Type Culture Collection (No. CCL 81)
SK-N-SH	Courtesy of Dr. Simon Swingler, University of Warwick.
Escherichia Coli (TG2)	Courtesy of Dr. Jaspel Randhawa, University of Warwick.
GMEM	Prepared by the media preparation unit, University of Warwick, Coventry.
DMEM	Prepared by the media preparation unit, University of Warwick, Coventry.
2x DMEM	Prepared by the media preparation unit, University of Warwick, Coventry.
2% Agar	Prepared by the media preparation unit, University of Warwick, Coventry.
Foetal Calf Serum	Sigma
Glutamine	Prepared by the media preparation unit, University of Warwick, Coventry.
Penicillin/Streptomycin	Prepared by the media preparation unit, University of Warwick, Coventry.
Tissue Culture Flasks	Nunc
6 Well Plates	Nunc
EDTA/Trypsin	GIBCO BRL

Trypsin/versene	Prepared by the media preparation unit, University of Warwick, Coventry.
Phosphate Buffered Saline	Prepared by the media preparation unit, University of Warwick, Coventry.
Crystal Violet	Sigma

II.2.2 Viruses

Respiratory Syncytial Virus (RSS-2)	Courtesy of Dr. Patricia Cane, University of Warwick, Coventry.
Measles Virus (Edmonston)	Courtesy of Professor Craig Pringle, University of Warwick, Coventry.
SSPE Virus (Yamagata-1)	Courtesy of Professor Homma, Yamagata University School of Medicine, Japan

II.2.3 Chemicals

II.2.3.1 BDH Laboratory Supplies

Acetic Acid

Boric Acid

Bromophenol Blue

Chloroform ($CHCl_3$)

Dimethylformamide

Glycerol

Magnesium Chloride ($MgCl_2$)

Magnesium Sulphate ($MgSO_4$)

Potassium Acetate (KAc)

Sodium Acetate (NaAc)

Sodium Chloride ($NaCl_2$)

Sodium Hydroxide (NaOH)

Tris(hydroxymethyl)methylamine

Xylene-Cyanol FF

II.2.3.2 Biorad

N,N,N',N'-Tetramethylethylenediamine

II.2.3.3 DIFCO Laboratories

Tryptone

Bactoagar

II.2.3.4 Fisons

Acrylamide

Ammonium Persulphate

Calcium Chloride ($CaCl_2$)

Diaminoethanetetra-Acetic Acid Disodium Salt (EDTA)

Ethanol

Glucose

Hydrochloric Acid (HCl)

Hydrogen Peroxide (H_2O_2)

Methanol

Paraffin Oil

Phenol

Potassium Chloride (KCl)

Sodium Dodecyl Sulphate ($C_{12}H_{25}OSO_3.Na$)

Urea

II.2.3.5 GIBCO BRL

Agarose

1kb DNA Ladder

Ligase

Restriction Enzymes:	Bam HI
	Hind III
	Kpn I
	Pst I
	Pvu II
	Sal I
Superscript II	
Thermus aquaticus (Taq) DNA Polymerase	

II.2.3.6 Miscellaneous

Bacteriophage M13	Courtesy of Mr. Kurt Tolley, University of Warwick, Coventry.
Formamide	Fluka Chemica
Geneclean Kit	BIO 101
pGEMT	Promega
Polyethylene Glycol (PEG) 6000	Prolabo
Primers	Prepared at University of Warwick , Coventry.
Sequenase Sequencing Kit	United States Biochemicals
Taq Cycle Sequencing Kit	United States Biochemicals
Vent DNA Polymerase	New England Biolabs
X-Gal	Rotec

Yeast Extract	OXOID

II.2.3.7 Sigma

Diatomaceous Earth

Dithiothreitol (DTT)

Ethidium Bromide

II.2.3.8 Pharmacia

Deoxy-nucleotides

Dideoxy-nucleotides

II.2.4 Apparatus

II.2.4.1 Electrophoresis Apparatus

GIBCO BRL Model 400H

Life Technologies Inc. Horizon 11.14

Pharmacia Electrophoresis Apparatus GNA-100

Pharmacia LKB. ECPS 3000/150

Sequencing plates, spacers and well formers GIBCO BRL

II.2.4.2 Miscellaneous

Chromatography Paper	Whatman
373A DNA Sequencer	Applied Biosystems
Eppendorf Tubes	Sarstedt
Falcon Tubes	Becton Dickinson Labware
Incubators	Lab Impex Research
Orbital Shaker	New Brunswick Scientific
Petri Dishes	Sarstedt
Taq DyeDeoxy Terminator Cycle Sequencing Kit	Applied Biosystems
Vacuum Dryer	Hoefer Scientific Instruments
X-Ray Film	Fuji
Universals	Media

PART III
RESULTS

III.CHAPTER 1

AMPLIFICATION AND SEQUENCING OF THE MATRIX GENE OF RESPIRATORY SYNCYTIAL VIRUS

III.1.1 The Effect of Passage of Human Respiratory Syncytial Virus in Neuroblastoma Cells

Human respiratory syncytial virus (huRS virus) belongs to the family *Paramyxoviridae*. Unlike measles virus, however, it has not been shown to involve the CNS during infection. It has been hypothesised that biased hypermutation events within the genome of measles virus provide some selective advantage for the survival of this virus in the CNS (Cattaneo *et al*, 1988a). Such mutations may be caused by a cellular enzyme i.e., double-stranded RNA adenosine deaminase (DRADA), previously referred to as RNA unwinding/modifying enzyme (Bass *et al*, 1989). Propagation of an SSPE virus (Yamagata-1) in neuroblastoma cells revealed additional mutational events, particularly U-C transitions, when compared to virus propagated in cells of non-neural origin. It was suggested that the additional mutations occurred as a consequence of viral growth in the neuroblastoma cell lines (Wong *et al*, 1989). As huRS virus does not naturally infect neural cells, propagation of this virus in cells of neural origin should not induce any genetic changes if biased hypermutation is an intrinsic property of the viral genome. If, however, the mutations observed in the Yamagata-1 virus are due to host cell enzyme activity, huRS virus should also be affected. The following experiments were carried out in order to determine the effect of passage of huRS virus in neuroblastoma cells.

HuRS virus was passaged 10 times in two neuroblastoma cell lines (IMR-32 and SK-N-SH) and 10 times in human lung fibroblast cells (MRC-5) (II.2.2). The aim of this experiment was to determine if biased hypermutation occurred as a result of passage in the neuroblastoma cells, using virus passaged in the MRC-5 cells as the control. A

characteristic of SSPE is that little or no infectious measles virus can be detected in the brain tissue of such patients. If biased hypermutation is involved in the development of a persistent infection, and, thus, the change in phenotype of the virus from a lytic infection to a cell-associated infection, a drop in titre of the virus might be expected as the number of viral passages increases. The titre of huRS virus was determined during sequential passage in each of the three cell lines using plaque assays (II.2.4). Table 2 shows the titres of huRS virus after each passage.

Table 2. HuRS virus passage titres

	Virus Titre (10^5 pfu/ml ± SD)		
Passage Number	MRC-5	IMR-32	SK-N-SH
1	2.09±1.72	0.01±0.04	0.01±0.003
2	0.23±0.03	0.22±0.01	53.20±5.10
3	1.40±0.01	0.31±0.07	3.64±0.17
4	0.20±0.04	10.50±2.82	1.43±0.21
5	1.72±0.54	0.15±0.07	13.10±1.64
6	0.32±0.10	1.06±0.53	7.00±1.98
7	0.38±0.04	0.43±0.17	16.70±11.72
8	0.18±0.02	0.20±0.05	5.26±1.52
9	2.55±1.01	1.78±0.35	2.57±0.35
10	0.95±0.37	0.56±0.23	0.63±0.06

HuRS virus was passaged 10 times in two neuroblastoma cell lines (IMR-32 & SK-N-SH) and human lung fibroblast cells (MRC-5) (II.2.2). After each sequential passage the virus titre (10^5 pfu/ml ± standard deviation of the mean [SD]) was determined by plaque assay using Vero cells (II.2.4).

Initially huRS virus did not grow well in the neuroblastoma cells, the titres were approximately 40-fold lower than that of virus passaged once in MRC-5 cell. However, adaptation for growth in the neuroblastoma cell lines occurred by the second passage. The titres of huRS virus passaged twice in IMR-32 cells and in MRC-5 cells are comparable. The titre for huRS virus passaged twice in the SK-N-SH cells was much higher and remained higher than the titre of virus passaged in MRC-5 cells. There

appears to be a more regular cyclical nature to the titres of huRS virus propagated in MRC-5 cells, compared to virus passaged in the two neuroblastoma cell lines. Each increase in virus titre is followed by a drop in titre, occasionally to a lower titre than that determined after passage one i.e., passages 4 and 8. Human RS virus required 2 passages in both of the neuroblastoma cell lines to adapt, after which the virus titre fluctuations observed were similar to that seen in huRS virus passaged in the MRC-5 cell line.

III.1.1.1 Discussion

Pipetting errors during the plaque assay procedure and / or in the counting of the plaques could account for some of the variability observed in the virus titres. However, an alternative explanation involves the design of the experiment. Rather than using a standard titre of virus for each of the virus passages, a standard volume of virus was used as the inoculum instead. This resulted in a variable titre of virus added to each cell monolayer. Most viruses develop defective interfering (DI) particles if a multiplicity of infection greater than 1 pfu cell is used during propagation, although this has not been observed with many strains of huRS virus (Professor C.R. Pringle, personal communication). Although the virus titres were shown to increase throughout the passage in each of the 3 cell lines, the inoculum for the sequential passage was not diluted to minimise the production and accumulation of DI particles. The presence of DI particles would interfere with the replication and release of infective virus so reducing the titre of the next passage, as such, the cyclical fluctuation in the titres observed could have been a consequence of fluctuation in the amount of DI particles present.

Nonetheless, adaptation for growth of huRS virus in the two neuroblastoma cell lines was observed as the titre determined for passage 10 in IMR-32 cells and SK-N-SH cells is higher than that for passage one in the respective cell lines. However, conclusive evidence for adaptation for growth in these cell lines would require determination of the efficiency of plating (EOP) of the virus in these cells. The aim of this experiment was not to determine specifically whether huRS virus would need a period of adaptation for growth in neuroblastoma cell lines, but to observe any change in phenotype of the virus which may occur during propagation in each of the 3 cell lines used. It was, therefore, not considered necessary to determine the EOP of the virus in each of the cell lines used.

Biased hypermutation is associated with SSPE viruses, which are a persistent non-lytic form of measles virus. Development of persistence of measles virus in the CNS is thought to occur as a consequence of biased hypermutation, along with other cellular factors. If huRS virus persistence follows the passage of virus in neural cells, this may be indicative of a potential for biased hypermutation. The overall virus titre of huRS virus propagated in the two neuroblastoma cells did not fall, therefore, it is unlikely that a transition from a lytic phenotype to a more persistent phenotype was occurring. However, the development of persistence may require greater than 10 passages, thus, the experimental design did not favour the development of a persistent infection. The huRS virus infected cell monolayers were harvested by mechanical means when approximately 50-75% of the monolayer was showing cytopathic effect. It is possible that any cell-associated virus present would be destroyed along with the monolayer, therefore, released rather than cell-associated virus was propagated further.

III.1.2 Amplification by Polymerase Chain Reaction of the M Gene of HuRS Virus

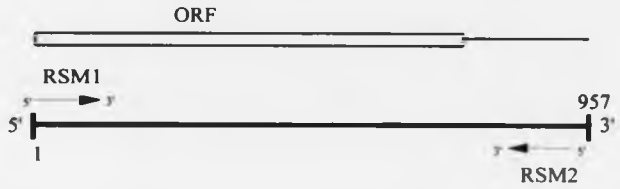
Total RNA was extracted from the huRS virus infected cells. mRNA was reverse transcribed using initially Avian Myeloblastosis Reverse Transcriptase (AMV-RT), then Superscript II, along with an oligo dT primer producing negative sense cDNA. The matrix (M) gene of huRS virus was then amplified by polymerase chain reaction (PCR) using *Thermus aquaticus* (Taq) polymerase (II.3.3.1). The published sequence of the matrix gene of huRS virus strain A2 (Satake and Venkatesan, 1984) was used to design the primers for the amplification as the M gene of the RSS-2 strain of huRS virus had not been sequenced. Each primer was designed to include unique restriction endonuclease (RE) cleavage sites in order to aid any subsequent cloning procedures. The same set of PCR primers was used to amplify M gene sequences from virus passages 1 and 10 in each of the three cell lines. The primers used and their amplified PCR products are shown below (Table 3 and Figures 10 & 11). The amplified fragment was 917 base pairs in size.

Table 3. PCR primers for the M gene of huRS virus

Name	Position	Sequence	ER Sites	Tm °C
RSM1	1 to 22	5' G GGT **ACC CGG G**GC AAA TAT GGA AAC ATA CG 3'	Sma 1	64
RSM2	917 to 900	3' GGG ATC **CTG CAG** ACT TGG GAT GAT CTG GG 5'	Pst 1	56

Endonuclease restriction (ER) sites are indicated by bold-type lettering. Two ER sites were included in each primer. However, efficient cleavage does not occur if the ER site is at the end of the primer, therefore, the internal ER site would be used for cloning reactions. A melting temperature of >54°C was employed to improve the specificity of the annealing reaction.

Figure 10. Position of the PCR primers within the M gene of huRS virus



The primers were designed so that the complete open reading frame (ORF) of the matrix gene could be amplified.

Figure 11. HuRS virus M gene fragments amplified by PCR

L: 1kb ladder (GIBCO BRL); 1: negative control (no cDNA); 2 & 5: passages 1 & 10 in IMR-32 cells respectively; 3 & 6: passages 1 & 10 in SK-N-SH cells, respectively; 4 & 7: passages 1 & 10 in MRC-5 cells, respectively. Half of the PCR product (50 µl) was electrophoresed on a 1% agarose TAE gel. The fragment was excised from the gel and purified using the Geneclean kit (BIO 101) (II.5.1). The purified fragment could then be sequenced (II.8.1.1 & II.8.1.2). The 180 bp fragment may represent non-specific binding of the primers to the template.

III.1.3 Sequencing the M Gene of HuRS Virus

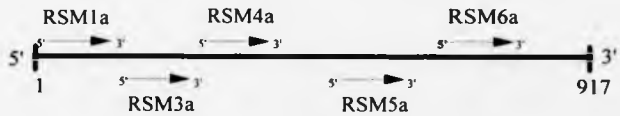
The reverse-transcribed, PCR amplified M gene of huRS virus passaged once and 10 times, in each cell line respectively, was sequenced. The M gene sequence of RSS-2 virus was established from the sequence obtained from virus passaged once in each cell line. This sequence was then used for comparison with sequence data obtained from virus passaged 10 times in order to determine whether any mutational events had occurred during propagation of the virus in each cell line. The amplified huRS virus M gene fragments were subjected to Geneclean (BIO 101) procedures and then sequenced directly using the US Biochemicals Taq Polymerase Cycle Sequencing Kit and the Applied Biosystems 373A automated sequencer (operated by Lesley Ward). Five internal sequencing primers were designed for the Taq polymerase cycle sequencing method, and 3 additional internal sequencing primers for the 373A automated sequencing method. The primers for the automated sequencer were designed before the method of manual sequencing had been decided. These primers could not be used for Taq polymerase cycle sequencing which requires the primer to be extended in order to incorporate the label (^{35}S-dATP). The primers designed for the automated sequencer would not incorporate sufficient label to give a strong signal on the gel (II.8.1.2), and as a consequence a further set of primers had to be designed. The primers used for sequencing and their positions within the M gene can be seen in Tables 4 and 5, and in Figures 12 and 13. The sequence obtained was positive sense (5' to 3') cDNA, which is identical to the mRNA sequence except that the thymine bases will be uracil in the RNA species.

Table 4. Sequencing primers for Taq polymerase cycle sequencing

Name	Position	Sequence	Tm °C
RSM1a	nt. 22 to 44	5' GTG AAC AAG CTT CAC GAA GGC TC 3'	70
RSM3a	nt 207 to 226	5' GGG ACC TTC ACT AAG AGT CAT G 3'	66
RSM4a	nt 346 to 371	5' GGC ATG TAG TCT AAC ATG CCT AAA AT 3'	72
RSM5a	nt 526 to 551	5' CAC ACT TGA AAA TAT AAC AAC CAC TG 3'	70
RSM6a	nt 693 to 718	5' GTA TAT ATT ATG TTA CCA CAA ATT GG 3'	66

The sequencing primers were designed from the huRS virus strain A2 M gene sequence (Satake & Venkatesan, 1984). They were designed to produce sequence in the 5' to 3' direction (+ sense). The primer extension region was designed such that a minimum of 5 ^{35}S-deoxy-adenosine residues would be incorporated.

Figure 12. Position of the sequencing primers for Taq polymerase cycle sequencing



The sequencing primers were positioned so that sequencing data from the most downstream primer would run through the next upstream primer. Cycle sequencing reactions generally produce 150-250 bases of sequence. Therefore, the primers have been placed approximately 150 to 200 bases apart.

Table 5. Sequencing primers for the 373A automatic sequencer

Name	Position	Sequence	Tm ^{o}C
RSM1	nt 1 to 22	5' GGG GCA AAT ATG GAA ACA TAC G 3'	64
RSM3	nt 239 to 253	5' GAA GTG GAC TGC TAG 3'	50
RSM4	nt 495 to 510	5' GAT CCA TCA GTG TCA G 3'	52
RSM5	nt 717 to 734	5' GGA AGC ACA CAG CTA CAC 3'	60

The primers for automated sequencing were also designed from the M gene of the A2 strain of huRS virus (Satake & Venkatesan, 1984). The melting temperature of the primers had to be ≥ 50^{o}C as this was the annealing temperature used by the operator of the machine.

Figure 13. Position of the sequencing primers for the 373A automated sequencer

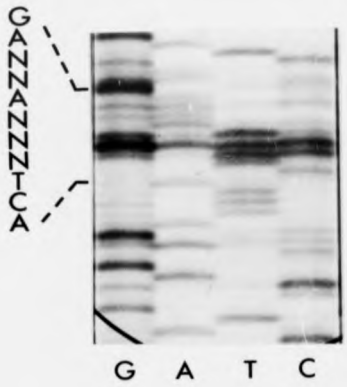
As before, these primers were positioned so that the sequencing data from the most downstream primer would run through to next stream primer. Automated cycle sequencing generally produced 250-400 bases of sequence depending on the quality of the DNA in the sample. Therefore, these sequencing primers were positioned approximately 200-250 bases apart.

From Figures 12 and 13 it can be seen that the sequencing primers are facing in the same direction i.e., 5' to 3'. As the PCR product is double-stranded another set of primers could have been used which would have enabled the second strand of the PCR product to be sequenced. Determining the sequence of both strands of the PCR product would make it easier to distinguish polymerase errors generated during the primer extension or cycle amplification of the M gene, from genuine base changes when compared to the M gene of the A2 strain of huRS virus and errors generated during the reverse transcriptase-PCR step. However, the absence of significant changes in the M gene sequence as a consequence of passage in neuroblastoma cells did not warrant the additional expenditure

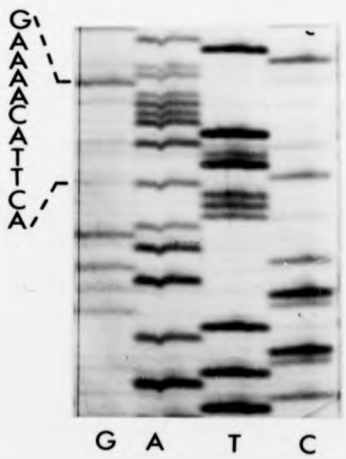
on additional primers. The sequence data from the cycle sequencing reactions is generally more ambiguous than that from a clone. Often signals of equal intensity occur at a particular position making it impossible to assign the correct base. Had the second strand of the PCR product been sequenced these ambiguities would have been resolved more easily. As this method was not employed an additional sequencing reaction had to be carried out using a different aliquot of the PCR product and a second gel electrophoresed to verify the sequence. This was a more laborious approach which resulted in more gels being processed than was really necessary. When the a 'problem' base had been identified it had to be confirmed either by manual sequencing or automated sequencing. Figures 14 and 15 show base determinations using both methods of sequencing.

Figure 14. Determination of sequencing ambiguities using Taq polymerase cycle sequencing

A.



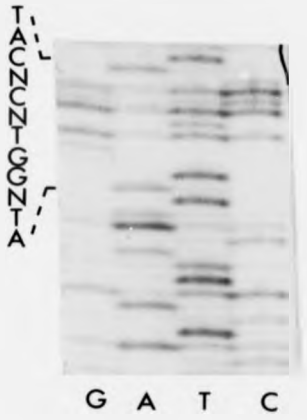
B.



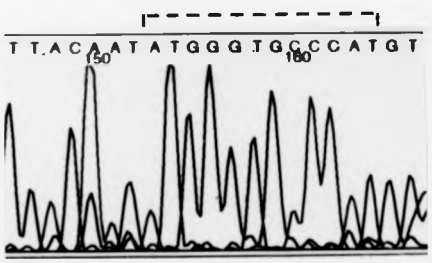
The sequencing reaction is from huRS virus passaged 10 times in MRC-5 cells. Taq polymerase cycle sequencing reaction was carried out according to the manufacturer's protocol. The primer RSM1a was used. A 2.5 µl aliquot of the reaction was electrophoresed on a polyacrylamide gel for 1.5-2 hrs (II.4.2). A: autoradiograph showing undetermined bases at positions 154, 155, 157-159. B: autoradiograph showing the determined bases at positions 154, 155, 157-159.

Figure 15. Determination of sequence ambiguities using automated sequencing

A.



B.



The sequence is from huRS virus passaged once in SK-N-SH cells. A. Taq polymerase cycle sequencing was used according to the manufacturer's protocol, along with the RSM1a primer. A 2.5 µl aliquot of the reaction was electrophoresed on a polyacrylamide gel (II.4.2). The undetermined bases are at positions 111, 113 and 119. B. One microgram of DNA and 3.2 pmol of RSM1 primer were made up to a final volume of 10.5 µl. This was then sent to the operator of the automated sequencer. Bases at positions 111, 113 and 119 have been determined.

III.1.4 The Sequence of the HuRS Virus M Gene

III.1.4.1 The Sequence of the RSS-2 M Gene Determined from Virus Passaged Once in MRC-5, IMR-32 and SK-N-SH Cells

The M gene sequence derived from huRS virus passaged once in the three cell types, MRC-5, IMR-32 and SK-N-SH, was sequenced at least twice to ensure any base changes observed were not due to sequencing errors. The M gene sequence derived from huRS virus passaged once in each of the 3 cell lines was found to be identical. This sequence could then be used for comparison with M gene sequence data obtained from huRS virus passaged 10 times in each of the 3 cell lines. Comparison of the M gene sequence of strain RSS-2 with that of the A-2 strain (Satake and Venkatesan, 1984) revealed a total of 30 base changes. Seventeen of the changes were within the coding region; however, they did not result in any amino acid replacements. The remaining 13 bases changes were located in the non-coding region (Figure 16). Of the 30 differences present, 24 represent transitions, 16 of which were either U-C or C-U; the remaining 6 changes were transversion, resulting in a ratio of 4:1 of transitions over transversions. The first 22 nucleotides of the M gene of huRS virus in each of the virus passages was not determined because this region was defined by the sequence of the 5' primer.

Homology of the matrix gene of the RSA-2 and RSS-2 strain at the nucleotide level was 96.83%. At the predicted amino acid level homology between the M protein of the two huRS virus strains was 100%.

Figure 16. Nucleotide sequence comparison of the M gene of the RSA-2 and RSS-2 strains of huRS virus

```
RSA-2 AUG GAA ACA UAC GUG AAC AAG CUU CAC GAA GGC UCC ACA UAC ACA     54
RSS-2                   .. ... ..A ... ... ... ... ... ... ... ...

RSA-2 GCU GCU GUU CAA UAC AAU GUC UUA GAA AAA GAC GAU GAC CCU GCA     99
RSS-2 ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...

RSA-2 UCA CUU ACA AUA UGG GUG CCC AUG UUC CAA UCA UCU AUG CCA GCA    144
RSS-2 ... ... ... ... ... ... ... ... ... ... ... ..C ... ... ...

RSA-2 GAU UUA CUU AUA AAA GAA CUA GCU AAU GUC AAC AUA CUA GUG AAA    189
RSS-2 ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...

RSA-2 CAA AUA UCC ACA CCC AAG GGA CCU UCA CUA AGA GUC AUG AUA AAC    234
RSS-2 ... ... ... ... ... ... ... ... ... U.. ... ... ... ... ...

RSA-2 UCA AGA AGU GCA GUG CUA GCA CAA AUG CCC AGC AAA UUU ACC AUA    279
RSS-2 ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...

RSA-2 UGC GCU AAU GUG UCC UUG GAU GAA AGA AGC AAA CUA GCA UAU GAU    324
RSS-2 ..U ..C ... ... ... ... ... ... ... ... ..G ..G ... ... ...

RSA-2 GUA ACC ACA CCC UGU GAA AUC AAG GCA UGU AGU CUA ACA UGC CUA    369
RSS-2 ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...

RSA-2 AAA UCA AAA AAU AUG UUG ACU ACA GUU AAA GAU CUC ACU AUG AAG    414
RSS-2 ... ... ... ... ... ..A ... ... ... ... ... ... ... ... ..A

RSA-2 ACA CUC AAC CCU ACA CAU GAU AUU AUU GCU UUA UGU GAA UUU GAA    459
RSS-2 ... ... ... ..A ... ... ..C ..C ... ... ... ... ... ... ...

RSA-2 AAC AUA GUA ACA UCA AAA AAA GUC AUA AUA CCA ACA UAC CUA AGA    504
RSS-2 ..U ... ... ... ... ... ... ... ... ... ... ... ... ... ...

RSA-2 UCC AUC AGU GUC AGA AAU AAA GAU CUG AAC ACA CUU GAA AAU AUA    549
RSS-2 ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
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RSA-2 ACA ACC ACU GAA UUC AAA AAU GCU AUC ACA AAU GCA AAA AUC AUC    594
RSS-2 ... ... ... ... ... ... ... ..C ... ... ... ... ... ... ...

RSA-2 CCU UAC UCA GGA UUA CUA UUA GUC AUC ACA GUG ACU GAC AAC AAA    639
RSS-2 ... ... ... ... ... ..G ... ... ... ... ... ... ... ... ...

RSA-2 GGA GCA UUC AAA UAC AUA AAG CCA CAA AGU CAA UUC AUA GUA GAU    684
RSS-2 ... ... ... ... ... ... ... ... ... ... ... ..U ... ... ...

RSA-2 CUU GGA GCU UAC CUA GAA AAA GAA AGU AUA UAU UAU GUU ACC ACA    729
RSS-2 ... ... ... ... ... ... ... ... ... ... ... ... ... ..A ...

RSA-2 AAU UGG AAG CAC ACA GCU ACA CGA UUU GCA AUC AAA CCC AUG GAA    774
RSS-2 ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...

RSA-2 GAU UAA CCUUUUUCCUCUACAUCAGUGUGUUAAUUCAUACAAACUUUCUACCUACAU    831
RSS-2 ... ... ........U............A...G.......................

RSA-2 UCUUCACUUCACCAUCACAAUCACAAACACUCUGUGGUUCAACCAAUCAAACAAAACUU    890
RSS-2 ............A....U......C...C.............U............CC

RSA-2 AUCUGAAGUCCCAGAUCAUCCCAAGUC                                    917
RSS-2 .....G..C.U.............C
```

The M gene sequence of the RSS-2 strain of huRS virus was determined by Taq polymerase cycle sequencing (USB), following the manufacturer's protocol; or by 373A automated cycle sequencing. A consensus sequence was determined by comparing sequence obtained from huRS virus passaged once in IMR-32 cells, SK-N-SH cells and MRC-5 cells. The sequence is displayed as mRNA (5' to 3' sense). However, the sequence determined was positive sense cDNA, which is identical to the mRNA of the M gene except that uracil residues would read as thymine on the sequencing gels.

AUG: translation start codon
UAA: translation stop codon

III.1.4.2 Comparison of the Nucleotide Sequence of the M Gene of the RSS-2 Strain of HuRS Virus Passaged Once and Ten Times in MRC-5, IMR-32 and SK-N-SH Cells

The M gene sequences of the RSS-2 strain of huRS virus passaged 10 times in each of the three cell lines, MRC-5 (human lung fibroblast cells), IMR-32 and SK-N-SH (both neuroblastoma cells), were compared with the sequence for the M gene of RSS-2 determined from virus passaged once in each of the 3 cell lines (III.1.4.1). No additional base changes were revealed.

III.1.4.3 Discussion

Limited propagation of huRS virus in neuroblastoma cell lines did not result in the accumulation of mutations in the M gene. Adenosine to guanine (A-G) hypermutations in the genomic sense (U-C in mRNA) have been identified in two escape mutants of huRS virus in the G gene (Rueda *et al.*, 1994). It was suggested that these transitions had occurred via the double-stranded RNA adenosine deaminase (DRADA) activity, although this has not been proven. The fact that escape mutants have been isolated which do contain U-C mutations does suggest that the viral genome may be susceptible to biased hypermutations, and so possibly a substrate for DRADA.

The experiment described in this thesis shows that hypermutation is not seen in the predominant M gene population. Preparing a cDNA library and screening for M gene clones using hybridisation techniques would give a more representative view of the M

gene population and would reveal whether hypermutation does occur in a small sub-population. However, using an M gene oligonucleotide as a probe is likely to result in the identification many potentially positive clones. Screening each of these clones by sequencing would have been very time consuming. Although such a method may result in a more realistic representation of the M gene population, it would have taken too long with respect to the time constraints of a 3 year research project, considering that ADD-MV passaged material would have to be treated in the same way. It was considered, therefore, adequate to sequence the PCR product. An advantage to sequencing the PCR product is that any errors in the sequence which are a result of polymerase mis-incorporation during the M gene amplification step can be visualised. Such errors will show on the autoradiograph as signals of equal intensity in each of the 4 nucleotide tracks (Figure 14a). Polymerase errors which occur at later stages during the amplification of the M gene will not be perpetuated and so should not influence the sequence of the predominant M gene population. However, a disadvantage of sequencing the PCR product is that minor sequences will not be amplified as readily and will not, therefore, contribute to the sequence.

Although the initial virus stock used was not plaque purified, the sequence obtained represents that of the majority component of the population. The data indicate that there was no significant change in the predominant component of the population as a consequence of passage. There is no evidence to suggest, therefore, that passage of plaque-purified virus would have resulted in the observation of hypermutated M gene species.

III.CHAPTER 2

AMPLIFICATION AND SEQUENCING OF THE MATRIX GENE OF ACUTE-DISEASE DERIVED MEASLES VIRUS

III.2.1 The Effect of Passage of Acute-Disease Derived Measles Virus in Neuroblastoma Cells

The Edmonston strain of acute-disease derived measles virus (ADD-MV) was passaged 10 times in 4 cell lines; IMR-32 and SK-N-SH (both human neuroblastoma cell lines), MRC-5 cells (human diploid lung fibroblast cells) and Vero cells (monkey kidney epithelial cells). The virus stock ($6x10^5$ pfu/ml) was diluted 1:100 to give a titre of $6x10^3$ pfu/ml, and 0.25 ml of this was used to infect a 25 cm^2 flask of cells (approximately $1.9x10^6$ cells) (II.2.1). The virus from passage 1 was harvested and diluted 1:100, 0.25 ml of this was used as the inoculum for passage 2, and so on. For each passage the same volume of diluted virus, rather than the same number of plaque forming units per cell, was added to the cells. The virus stock was diluted in order to reduce the inhibitory effect on replication of any DI particles which may have been present in the original inoculum or generated during passage. Progeny virus was harvested at 6 to 7 days post infection. Determining the optimum time of harvest of virus passaged in the neuroblastoma cell line was difficult as syncytia were rarely visible. The time of harvest was determined by the amount of cell death observed. Virus was harvested from the neuroblastoma cell cultures when approximately 50 - 75% of the cell monolayer became detached. For cultures in which syncytia were seen (Vero and MRC-5 cells), virus was harvested when the monolayer was showing 50 - 75% syncytia. The neuroblastoma cell line IMR-32 was difficult to grow. These cells grew slowly and cultures were vulnerable to infection with bacteria and yeasts. The addition of kanamycin and fungizone to the medium had a detrimental effect on the growth of these cells, such that passage and titration of the virus in this cell line was difficult. The virus passages from each cell line were titred on Vero

cells. The titres of the ADD-MV passaged 10 times in each of the 4 cell lines are shown in Table 6.

Table 6. ADD-MV passage titres

	Virus Titre ($x10^5$ ± SD pfu/ml)			
Passage No.	Vero	MRC-5	IMR-32	SK-N-SH
1	6.80±1.60	0.06±0.01	0.23±0.01	0.17±0.04
2	3.50±0.31	0.22±0.07	1.48±0.06	0.23±0.03
3	4.36±2.60	0.45±0.02	0.02±0.003	0.33±0.02
4	11.6±0.79	2.08±0.45	0.06±0.003	0.49±0.03
5	4.46±1.91	2.53±0.71	0.04±0.001	0.56±0.04
6	0.20±0.14	16.6±7.50	0.39±0.06	1.82±0.08
7	0.02±0.007	5.05±2.76	3.65±1.28	2.20±0.28
8	0.02±0.01	1.02±0.03	1.10±0.86	3.88±2.09
9	0.77±0.23	0.13±0.04	1.82±0.44	0.23±0.18
10	0.19±0.02	0.26±0.03	1.23±0.95	0.92±0.17

ADD-MV stock was diluted 1:100 and 0.25 ml of this was used to inoculate a 25 cm^2 flask of each of the 4 cell lines. Virus was harvested by mechanical destruction of the monolayer and the resulting virus progeny was diluted 1:100 and used as the inoculum for further passage (II.2.1). Each passage was titred on Vero cells as the neuroblastoma cell lines did not produce syncytia, thus titre determination using these cells was difficult. SD: standard deviation of the mean.

The original virus stock was inoculated into and subsequently passaged in human diploid cells only. No adaptation would be expected during further passage of ADD-MV in this cell line. Nonetheless, the titres of ADD-MV passaged in MRC-5 cells increased to a peak at passage 6 ($16.60x10^5$ pfu/ml). The standard deviation of the mean for this passage is quite high. This could be due to the high number of plaques present in the wells corresponding to the lower dilutions used in the virus titre protocol. Due to the difficulty in counting dense populations of plaques, only a small portion of the monolayer is counted. The total number of plaques from that dilution is determined by multiplying up i.e., if plaques from a quarter of the monolayer was counted, then that number would be multiplied by 4 to give the total number of plaques for that dilution. This method

amplifies any error made in the counting, which would account for the high standard deviation calculated. The titre of virus MRC-5 cells then fell to $0.13x10^5$ pfu/ml, before slightly rising to $0.26x10^5$ pfu/ml. The subsequent fall in titre could have been in response to an increasing concentration of defective interfering (DI) particles.

Vero cells are permissive for measles virus infection and there did not appear to be any adaptation for viral growth in this cell line. The virus titres throughout the 10 passages fluctuated greatly. Again, this could be due to the variable presence of DI particles in the inoculum.

ADD-MV had not previously been propagated in neuroblastoma cells, therefore some adaptation for growth in these cell lines might be expected. The titres from passage one in SK-N-SH cells and IMR-32 cells are low, but not as low as the titre for passage one in MRC-5 cells. The titres from sequential passages were determined with peak titres in IMR-32 cells at passage 7, and in SK-N-SH cells at passage 8.

Some of the fluctuation in virus titres observed could be due to pipetting errors during the plaque assay procedure, or counting error. More consistent results might have been obtained if a standard multiplicity of infection (MOI) rather than an arbitrary dilution had been used.

III.2.1.1 Conclusions

It has been suggested that DI particles play a role in the establishment of persistent infections (Huang & Baltimore, 1970). The passaged virus used in these experiments was diluted 1:100 in order to reduce the concentration of DI particles in the inoculum, however, it has been shown that virus stocks of measles virus should be diluted 1:10,000 in order to obtain measles virus preparations that are minimally contaminated with DI particles (Rima *et al.*, 1977). The MOI to produce such measles virus preparations was not evaluated, however, the titres of measles virus preparations used in the study of Rima *et al.* (1977) were approximately $1\text{-}4x10^{5}$ pfu/ml, on monolayers containing approximately $5x10^{6}$ cells. Therefore, the MOI of virus diluted $1:10^{4}$ was approximately $2\text{-}8x10^{-6}$ pfu/cell. The original inoculum used in the experiments described in this thesis had a titre of $6x10^{3}$ pfu/ml, which was diluted 1:100 and only 0.25 ml was used to infect a monolayer containing approximately $1.9x10^{6}$ cells, therefore the MOI used was $3x10^{-5}$ pfu/cell. This dilution factor should have reduced the probability of interference by DI particles.

There is no overall fall in the titre of ADD-MV in MRC-5 cells, nor virus passaged in IMR-32 and SK-N-SH cells. When comparing the virus titres of ADD-MV passaged in the neuroblastoma cell lines, with those determined from virus passaged in the MRC-5 cells, there is no obvious difference in the range of titres observed. ADD-MV did not required any adaptation for growth in the MRC-5 cells. The titres of virus passaged in the neuroblastoma cell lines are not significantly different when compared to virus passaged

in MRC-5 cells, which could suggest that no adaptation was required by the ADD-MV virus for propagation in the neuroblastoma cell lines.

III.2.2 Amplification by Polymerase Chain Reaction of the M Gene of ADD-MV

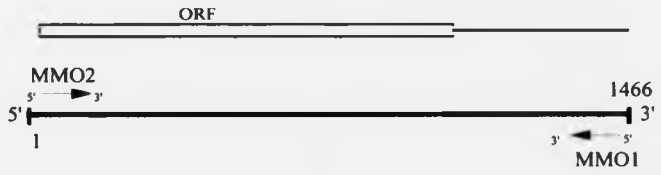
Total RNA was extracted from the infected cells of passages 1 and 10 in each of the cell lines. The RNA was then reverse transcribed initially using Avian Myeloblastosis Virus Reverse Transcriptase (AMV-RT), and subsequently using Superscript II, which became available in December 1994. The primer used for reverse transcription was the oligo dT primer, which would prime on the poly A tail of mRNA producing (-) sense cDNA. The matrix gene (M) of ADD-MV was then amplified by polymerase chain reaction (PCR) using *Thermus aquaticus* (Taq) polymerase. The published sequence of the M gene for the Edmonston strain of measles virus (Bellini *et al*, 1986), hereafter referred to as EdM, was used to design the primers for the amplifications. Each primer was designed to include endonuclease restriction (ER) cleavage sites to aid any subsequent cloning procedures. The aim of the experiment was to amplify the entire M gene of ADD-MV even though only the 5'-end 750 bases would be sufficient for the sequence comparisons (III.2.3). This region of the M gene was chosen because excess hypermutations observed in the SSPE virus (Yamagata-1) passaged in IMR-32 cells by Wong *et al.* (1989) occurred within the first 550 bases. Two primers MMO1 and MMO2 were designed for this purpose (Table 7) and their positions on the M gene are shown in Figure 17.

Table 7. PCR primers for ADD-MV M gene

Name	Position	Sequence	ER Sites	Tm °C
MMO1	1404 to 1387	3' **GGG ATC CAC** CTC GGT CGC TTG TGC 5'	Bam H1	60
MMO2	25 to 44	5' C **TCT CGA G**TT CCA CAA TGA CAG AGA TC 3'	Xho I	58

The primers were designed using the published M gene sequence of the Edmonston strain of measles virus (Bellini *et al*, 1986). Endonuclease restriction (ER) sites, indicated by bold-type lettering, were included at the 5' ends of each primer to aid any further cloning reactions. A melting temperature of ≥54°C was employed to improve the specificity of the annealing reaction.

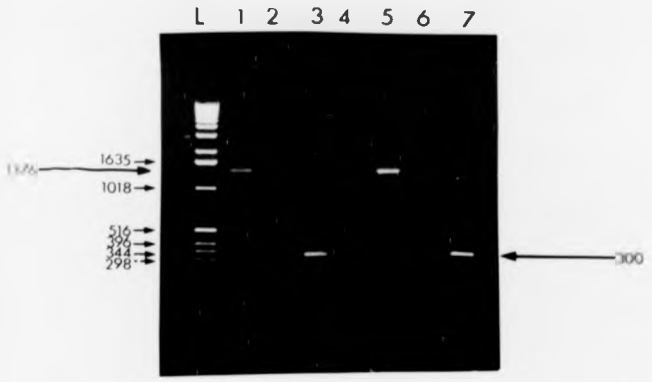
Figure 17. Position of the PCR primers within the M gene of ADD-MV



The primers were designed so that the entire M gene, minus a few bases in the non-coding regions at the 5' and 3' ends, could be amplified. The amplified fragment was expected to be 1379 bases.

Total RNA extracted from ADD-MV stock, plaque-purified in BS-C-1 cells (SL92/16/MV), was used to determine the most suitable cycle parameters for efficient amplification of the M gene. These were found to be 30 cycles of: 94°C for 45 secs, 56°C for 45 secs and 74°C for 2 mins. Figure 18 shows the amplified 1379 base M gene using the MMO1/MMO2 primer pair. Two primers designed to amplify a 300 base fragment of the NP gene of ADD-MV, MNO1 (108/ 5' AGA TGG CCA CAC TTT TAA GG 3' /127) and MNO2 (425/ 3' CTG GAC AAC CTC TAA CAG CC 5' /406) were used as controls for the PCR reaction. Transcription of genomic RNA occurs sequentially from the 3' terminus, which leads to a polar accumulation of mRNAs. The NP gene is the first gene to be transcribed and its mRNA will be more abundant. Therefore, amplification of a small region of this gene was chosen as the PCR control.

Figure 18. M gene amplification of SL92/16/MV using MMO1/MMO2 primers

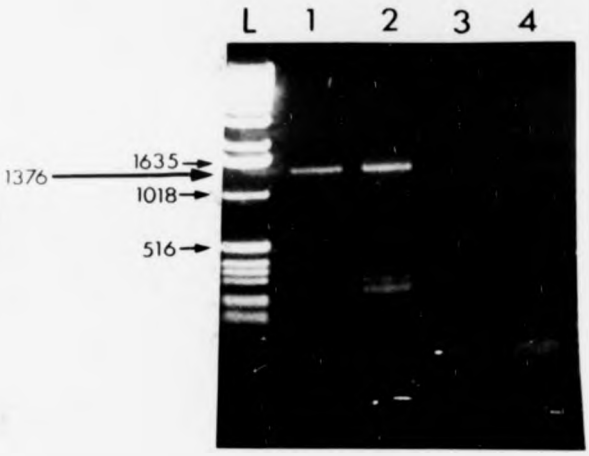


L: 1 kb ladder (GIBCO BRL); 1-4: PCR amplifications using 0.2 µl Taq polymerase; 5-7: PCR reactions using 0.4 µl Taq polymerase; 1+5: SL92/16/MV using MMO1/MMO2 primers; 2+6: mock infected BS-C-1 cells using MMO1/MMO2; 3+7: SL92/16/MV using MNO1/MNO2 primers; 4: mock infected BS-C-1 cells using MNO1/MNO2 primers. MNO1 and MNO2 primers were designed to amplify a 300 base fragment of the N gene, to be used as controls for the PCR reaction. The cycle parameters used were 94°Cx45 secs, 54°Cx45 secs and 74°Cx2 mins for 30 cycles. A 10 µl aliquot of each PCR product was subjected to electrophoresis on a 1% TBE agarose gel (II.4.1).

The 1379 base fragment of the M gene could be amplified using the MMO1/MMO2 primer pair (Figures 19A). However, in later experiments amplification of the M gene from ADD-MV passages, of both plaque purified virus and non-plaque purified virus, resulted in an 800 base fragment in place of the expected 1379 base fragment. To improve the stringency of the annealing reaction, the annealing temperature of the PCR cycle was increased to 58°C for 45 secs. and, as a result the 1379 base fragment was once again amplified (Figure 19B).

Figure 19. Amplification of the M gene of ADD-MV passaged once and 10 times using the MMO1/MMO2 primers

A.



B.

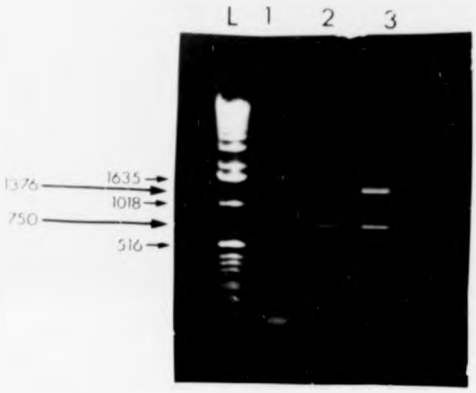
L: 1 kb ladder; A. 1: ADD-MV passaged once in SK-N-SH cells; 2: ADD-MV passaged once in IMR-32 cells; 3: ADD-MV passaged once in MRC-5 cells; 4: ADD-MV passaged once in Vero cells. Cycle parameter: 94°Cx45 secs, 54°Cx45 secs and 74°Cx2 mins (30 cycles). B. 1: mock infected SK-N-SH cells passaged 10; 2: ADD-MV passaged 10 times in SK-N-SH cells; 3: plaque purified ADD-MV passaged 10 times in SK-N-SH cells; 4: mock infected IMR-32 cells passage 10; 5: plaque purified ADD-MV passaged 10 times in IMR-32 cells. Cycle parameters: 94°Cx45 secs, 58°Cx45 secs and 74°Cx2 mins (30 cycles). A 10 µl aliquot of each PCR product was subjected to electrophoresis on a 1% TBE agarose gel. The smaller fragments (approx. 200 bp) may represent non-specific binding of the primers to the template.

Amplification of the expected 1379 base fragment was achieved consistently for a further 6 months, until, again, only a 750-800 base fragment of the M gene could be amplified using the MMO1/MMO2 primer pair in all passaged virus, irrespective of passage history or the passage number. This coincided with a change from using AMV-RT to Superscript II. It was possible that the change in reverse transcriptase may have affected the annealing specificity of the two primers. To test this, total RNA from cells infected with ADD-MV was extracted and resuspended in 5 μl of distilled water. Half of this material was reverse transcribed using Superscript II and the other half using AMV-RT. The resulting cDNA, prepared using the oligo dT primer, was amplified using the MMO1/MMO2 primers. Figure 20 shows that both the 1379 and the 750-800 base fragments were amplified regardless of the reverse transcriptase used, although the 1379 bp fragment amplified from cDNA synthesised using AMV-RT is faint. This result suggests that both primers were annealing to the intended sequences and that the 800 base fragment might represent a truncated version of the M gene. The virus used in this experiment was derived from the original ADD-MV used in the passage experiment, therefore, if the M gene was truncated then the original virus stock used for the virus passage would also contain the truncated M gene. Alternatively, one or both of the primers may have been mis-priming. However, since there is no obvious homology between other regions of the M gene and the two primers this seems an unlikely explanation for the amplification of the smaller M gene fragment.

In order to determine whether the 5'-end 750 bases required for this experiment had been amplified, the 800 base fragment was gel purified and sequenced by Taq polymerase cycle sequencing. The results of the sequencing experiments confirmed that the first 750

bases were present within the 800 base fragment, and the subsequent analysis was derived from sequencing this fragment.

Figure 20. Comparison of the PCR products from cDNA synthesised using AMV-RT and Superscript II

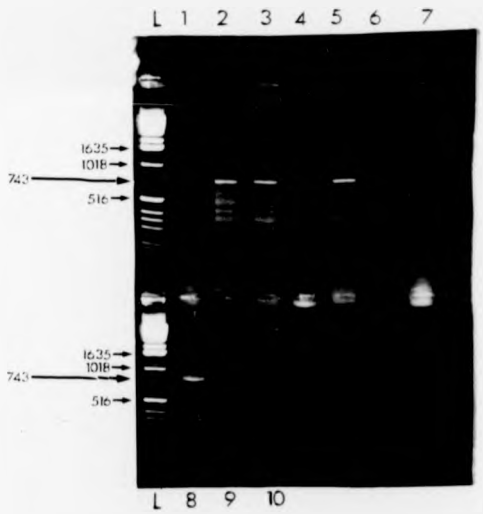


L: 1 kb ladder; 1: negative control (no cDNA); 2: ADD-MV (AMV-RT); 3: ADD-MV (Superscript II). Total RNA, extracted from ADD-MV infected cells, was reverse transcribed using AMV-RT and priming the mRNA with oligo dT, or Superscript II (II.3.2) and oligo dT. The resulting cDNA was amplified using MMO1/MMO2 primers. Cycle parameters: 94°Cx45 secs, 58°Cx45 secs and 74°Cx2 mins for 30 cycles. A 10 µl aliquot of the reaction was subjected to electrophoresis on a 1% TBE agarose gel.

Total RNA was extracted from stored aliquots of ADD-MV infected cells. Freshly diluted oligo dT primer was used for cDNA synthesis and primers MMO1/MMO2 used for PCR, along with new enzyme and buffer to determine whether degradation of cDNA or any of the materials used during the cDNA synthesis and PCR procedures affected the amplification of the 1379 base fragment. However, only the 800 base fragment could be amplified reproducibly. Occasionally the 1379 base fragment would be amplified, but

this was not reproducible on a regular basis. A new 5' primer was designed, MVM1 (1/ 5' AGG AGC AAA GTG ATT GCC TC 3' /20 Tm: 60°C). The aim was to use this primer in conjunction with the oligo dT primer to amplify the entire M gene (1466 bases) of ADD-MV. However, when the oligo dT/MVM1 primer pair were used together on both plaque purified virus and non-purified passaged virus a fragment of approximately 750 bases was amplified (Figure 21).

Figure 21. Amplification of ADD-MV using the MVM1/oligo dT primer pair



L: 1 kb ladder; 1: ADD-MV plaque purified (pp) once in MRC-5 cells; 2: ADD-MV passage 10 in SK-N-SH cells; 3: ppADD-MV passage 10 in SK-N-SH cells; 4: ADD-MV passage 10 in IMR-32 cells; 5: ppADD-MV passage 10 in IMR-32 cells; 6: ADD-MV passage 1 in MRC-5 cells; 7: ADD-MV passage 10 in MRC-5 cells; 8: ADD-MV passage 1 in Vero cells; 9: ADD-MV passage 10 in Vero cells; 10: ppADD-MV passage 1 in Vero cells. Cycle parameters used are 94°Cx45 secs, 54°Cx45 secs and 74°Cx2 mins for 30 cycles. A 10 µl aliquot of each PCR product was subjected to electrophoresis on a 1% TBE agarose gel.

From figure 21 it can be seen that several fragments of various sizes have been amplified. The most prominent fragment was that corresponding to approximately 750-800 bases which suggests that the oligo dT primer was annealing to the sequence 743/ 5' AAA ATG AAA ATC GAA AA 3' /759 in the M gene. Other smaller fragments and occasionally one of approximately 1 kb was observed, although these were less well defined. The amplification of smaller fragments could have been prevented by altering the cycle parameters. For example, the melting temperature of MVM1 and oligo dT is 60°C, therefore, the annealing temperature of the PCR cycle could be increased to 59°C. This would increase the fidelity of primer annealing so fewer fragments would be amplified and the PCR product would be expected to more specific. If the annealing temperature was increased it was possible that the oligo dT primer would not base pair with the 743-759 base region of the gene because 5 out of the 17 bases are miss-matched. There are other areas of the M gene where the oligo dT primer may have some homology e.g., nucleotide positions 1006-1034 and 1101-1121, but there was no other region where 12 out of 17 bases were complementary; therefore, it was possible to conclude that PCR amplification using the MVM1/oligo dT primer pair produced the 5'-end 759 bases of the M gene. As already stated, this was the region of the gene required for the sequence analysis, as such the amplification products of the MVM1/oligo dT primers were suitable for this experiment.

Essentially three PCR products of the M gene of passaged ADD-MV have been used in sequencing reactions. Initial PCR amplifications using the MMO1/MMO2 primer yielded the 1379 base fragment of the M gene which was expected and was subsequently used for sequence analysis of the 5' 750 bases. Later, only a 800 base fragment could be

generated. This was shown to contain the 5 -region of the M gene required for the sequence analysis and so these products were used for cycle sequencing reactions. Finally, the 743 base fragment amplified by the MVM1/oligo dT primers also yielded a product which could be used for sequencing.

The positions of all the primers used to amplify the M gene of ADD-MV are shown in Figure 22 below.

Figure 22. Nucleotide sequence of the ADD-MV M gene and the positions of the PCR primers

```
mRNA 5'AGGAGCAAAGUGAUUGCCUCCCAAGUUCCACAAUGACAGAGAUCUACGAC   50
  MVM1 AGGAGCAAAGTGATTGCCTC →
cDNA 3'TCCTCGTTTCACTAACGGAGGGTTCAAGGTGTTACTGTCTCTAGATGCTG
                          MM02 GTTCCACAATGACAGAGATC →

mRNA   UUCGACAAGUCGGCAUGGGACAUCAAAGGGUCGAUCGCUCCGAUACAACC   100
cDNA   AAGCTGTTCAGCCGTACCCTGTAGTTTCCCAGCTAGCGAGGCTATGTTGG

mRNA   CACCACCUACAGUGAUGGCAGGCUGGUGCCCCAGGUCAGAGUCAUAGAUC   150
cDNA   GTGGTGGATGTCACTACCGTCCGACCACGGGGTCCAGTCTCAGTATCTAG

mRNA   CUGGUCUAGGCGACAGGAAGGAUGAAUGCUUUAUGUACAUGUUUCUGCUG   200
cDNA   GACCAGATCCGCTGTCCTTCCTACTTACGAAATACATGTACAAAGACGAC

mRNA   GGGGUUGUUGAGGACAGCGAUCCCCUAGGGCCUCCAAUCGGGCGAGCAUU   250
cDNA   CCCCAACAACTCCTGTCGCTAGGGGATCCCGGAGGTTAGCCCGCTCGTAA

mRNA   UGGGUCCCUGCCCUUAGGUGUUGGCAGAUCCACAGCAAAGCCCGAAAAAC   300
cDNA   ACCCAGGGACGGGAATCCACAACCGTCTAGGTGTCGTTTCGGGCTTTTTG

mRNA   UCCUCAAAGAGGCCACUGAGCUUGACAUAGUUGUUAGACGUACAGCAGGG   350
cDNA   AGGAGTTTCTCCGGTGACTCGAACTGTATCAACAATCTGCATGTCGTCCC

mRNA   CUCAAUGAAAAACUGGUGUUCUACAACAACACCCCACUAACUCUCCUCAC   400
cDNA   GAGTTACTTTTTGACCACAAGATGTTGTTGTGGGGTGATTGAGAGGAGTG

mRNA   ACCUUGGAGAAAGGUCCUAACAACAGGGAGUGUCUUCAACGCAAACCAAG   450
cDNA   TGGAACCTCTTTCCAGGATTGTTGTCCCTCACAGAAGTTGCGTTTGGTTG

mRNA   UGUGCAGUGCGGUUAAUCUGAUACCGCUCGAUACCCCGCAGAGGUUCCCU   500
cDNA   ACACGTCACGCCAATTAGACTATGGCGAGCTATGGGGCGTCTCCAAGGGA

mRNA   GUUGUUUAUAUGAGCAUCACCCGUCUUUCGGAUAACGGGUAUUACACCGU   550
cDNA   CAACAAATATACTCGTAGTGGGCAGAAAGCCTATTGCCCATAATGTGGCT
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mRNA   UCCUAGAAGAAUGCUGGAAUUCAGAUCGGUCAAUGCAGUGGCCUUCAACC   600
cDNA   AGGATCTTCTTACGACCTTAAGTCTAGCCAGTTACGTCACCGGAAGTTGG

mRNA   UGCUGGUGACCCUUAGGAUUGACAAGGCGAUAGGCCCUGGGAAGAUCAUC   650
cDNA   ACGACCACTGGGAATCCTAACTGTTCCGCTATCCGGGACCCTTCTAGTAG

mRNA   GACAAUACAGAGCAACUUCCUGAGGCAACAUUUAUGGUCCACAUCGGGAA   700
cDNA   CTGTTATGTCTCGTTGAAGGACTCCGTTGTAAATACCAGGTGTAGCCCTT

                                                ←  TTTTTTT
mRNA   CUUCAGGAGAAAGAAGAGUGAAGUCUACUCUGCCGAUUAUUGCAAAAUGA   750
cDNA   GAAGTCCTCTTTCTTCTCACTTCAGATGAGACGGCTAATAACGTTTTACT

       TTTTTTTTTT Oligo dT
mRNA   AAAUCGAAAAGAUGGGCCUGGUUUUUGCACUUGGUGGGAUAGGGGGCACC   800
cDNA   TTTAGCTTTTCTACCCGGACCAAAAACGTGAACCACCCTATCCCCCGTGG

                              ----//---

mRNA   GCAGCCGACGGCAAGCGCGAACACCAGGCGGCCCCAGCACAGAACAGCC   1200
cDNA   CGTCGGCTGCCGTTCGCGCTTGTGGTCCGCCGGGGTCGTGTCTTGTCGG

mRNA   CUGACACAAGGCCACCACCAGCCACCCCAAUCUGCAUCCUCCUCGUGGGA   1250
cDNA   GACTGTGTTCCGGTGGTGGTCGGTGGGGTTAGACGTAGGAGGAGCACCCT

mRNA   CCCCCGAGGACCAACCCCCGGAGCUGCCCCCGAUCCAAACCACCAACCGC   1300
cDNA   GGGGGCTCCTGGTTGGGGGCCTCGACGGGGGCTAGGTTTGGTGGTTGGCG

mRNA   ACCCCCACCACCCCCGGGAAAGAAACCCCCAGCAAUUGGAAGGCCCCUCC   1350
cDNA   TGGGGGTGGGTGGGGCCCTTTCTTTGGGGGTCGTTAACCTTCCGGGGAGG

                                        ←  CGTCTTCGCTGGCT
mRNA   CCCUCUUCCUCAACACAAGAACUCCACAACCGAACCGCACAAGCGACCGA   1400
cDNA   GGGAGAAGGAGTTGTGTTCTTGAGGTGTTGGCTTGGCGTGTTCGCTGGCT

       CCAC MMO1
mRNA   GGUGACCCAACCGCGCGGCAUCCGACUCCCUAGACAGAUCCUCUCUCCCC   1450
cDNA   CCACTGGGTTGGCGCGCCGTAGGCTGAGGGATCTGTCTAGGAGAGAGGGG

mRNA   GGCAAACUAAACAAAA  3'                                 1466
cDNA   CCGTTTGATTTGTTTT  5'
```

mRNA:cDNA hybrid duplex formed during the reverse transcriptase reaction. M gene sequence determined by Bellini *et al.* (1986). Amplification primers (indicated by bold-type lettering) MMO2 and MVM1 anneal to the negative sense cDNA, while oligo dT and MMO1 anneal to the mRNA. The resulting amplified products should be MMO1/MMO2: 1379 base pairs; MVM1/oligo T: 743 base pairs.

III.2.3 Sequencing the 5'-end 750 Bases of the M Gene of ADD-MV

III.2.3.1 Sequencing the PCR Product

Initially, it was intended that the M genes of ADD-MV passaged once and 10 times would be sequenced in order to determine if biased hypermutation had occurred during propagation in cultured cells, and if this type of mutation occurred at a higher frequency in virus passaged in neuroblastoma cells compared to cells of non-neural origin. Had there been a high frequency of mutations in the M gene of ADD-MV passaged 10 times compared to that passage only once, the M gene from intermediate passages would have been sequenced to determine if the mutations occurred gradually during cell passage, or as a single event as suggested by Wong *et al.* (1989). As this would require a large amount of sequencing the method of preference was cycle sequencing. This sequencing method eliminated the need to clone the M genes into a Bluescribe vector, for double-stranded sequencing; or phage M13 for single-stranded sequencing, as the PCR product could be sequenced directly. The PCR products were gel purified then sequenced using both the US Biochemicals Taq Polymerase Cycle Sequencing Kit and the Applied Biosystems 373A automated sequencer.

In order to sequence the entire 5'-750 bases of the M gene it was necessary to design 3 internal sequencing primers for the Taq polymerase sequencing kit, and 3 internal sequencing primers for the 373A automated sequencer. The primers were designed from EdM (III.2.2), the resulting sequence will be (+) sense cDNA, which corresponds to the M gene mRNA sequence except that thymine bases in cDNA are uracil bases in mRNA.

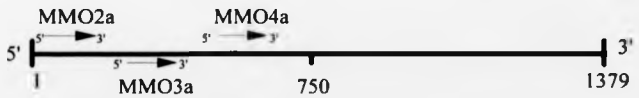
They were positioned within the gene so that sequencing data from the most downstream primer would run through the next upstream primer. The nucleotide sequence and position of the sequencing primers within the M gene are shown in Tables 8 and 9, and Figures 23 and 24.

Table 8. Sequencing primers for Taq polymerase cycle sequencing

Name	Position	Sequence	Tm °C
MMO2a	nt. 48 to 69	5' GAC TTC GAC AAG TCG GCA TGG G 3'	70
MMO3a	nt 267 to 287	5' GGT GTT GGC ACA TCC ACA GCA 3'	66
MMO4a	nt 530 to 553	5' GGA TAA CGG GTA TTA CAC CGT TCC 3'	72

The primers were designed using the published M gene sequence of the Edmonston strain of measles virus (Bellini *et al.*, 1886). They were designed so that at least 5 adenosine residues would be labelled during the primer extension reaction of cycle sequencing.

Figure 23. Position of the sequencing primers for Taq cycle sequencing



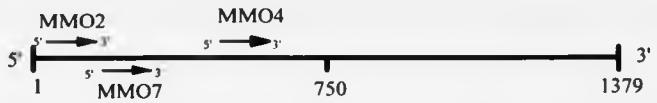
Approximately 200-250 bases of sequence can be determined from a 2 hr electrophoresis. Further runs of 3.5-4 hrs were necessary to produce sequence data that run from MMO2a through to MMO3a, and from MMO3a through to MMO4a

Table 9. Sequencing primers for the 373A automated sequencer

Name	Position	Sequence	Tm °C
MMO2	nt. 25 to 44	5' GTT CCA CAA TGA CAG AGA TC 3'	58
MMO4	nt. 552 to 567	5' CCT AGA AGA ATG CTG G 3'	48
MMO7	nt 134 to 151	5' GGT CAG AGT CAT AGA TCC 3'	54

Primers were designed using the published M gene sequence of the Edmonston strain of measles virus (Bellini *et al.*, 1986), and were designed such that sequence data from the most downstream primer would run through the next upstream primer.

Figure 24. Position of the sequencing primers for 373A automated sequencing



Approximately 250-400 bases can be determined using the automated sequencing machine depending on the quality of the DNA. Therefore the primers were positioned a maximum of 400 bases apart.

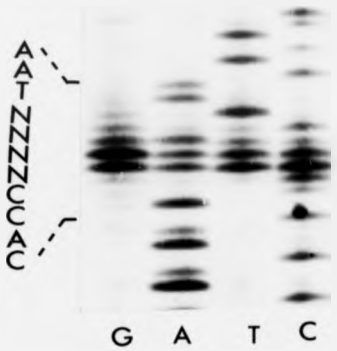
The sequencing primers for the 373A automated sequencer were designed before the method of manual sequencing had been determined. The primers required for cycle sequencing have to be positioned so that 10-20 bases from the annealing site of the primer can be extended with the incorporation of at least 5 ^{35}S-dATP bases. The primers for the automated sequencer were not positioned where labelling of the primer would result in the incorporation of enough ^{35}S-dATP label to produce a strong signal, and so new primers were designed.

The primers are all facing in the same direction, thus only one strand of the double-stranded PCR product was sequenced. Determining the sequence of both strands of the

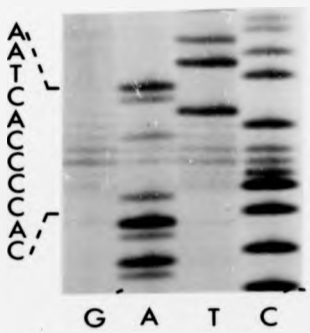
PCR product would have made it much easier to distinguish polymerase errors occurring as a result of the sequencing reaction, from genuine base changes when compared to the M gene of EdM. In order to resolve ambiguities the method of base determination used was to repeat the sequencing reactions and run a second gel, or to send DNA to the automated sequencer in order to verify which base was actually present (Figures 25 and 26). Once the base had been determined a third confirmatory reaction was carried out.

Figure 25. ADD-MV base determination using Taq cycle sequencing

A.



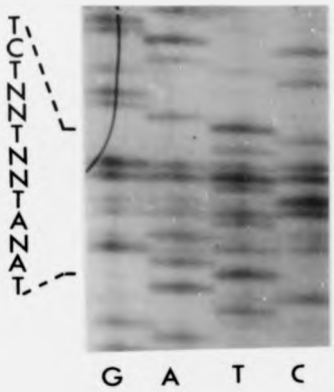
B.



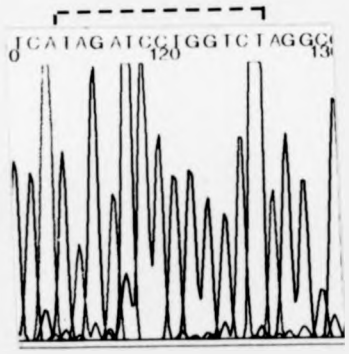
MMO3a primer sequencing reaction of ADD-MV passaged 10 times in IMR-32 cells. A: The following bases can not be determined from this reaction, 383-386. B: The sequencing reaction was repeated and the undetermined bases were resolved. The reactions were carried out following the manufacturer's protocol (II.8.1.2) and 2.5 µl was electrophoresed on a polyacrylamide gel (II.4.2).

Figure 26. ADD-MV base determination using the 373A automated sequencing machine

A.



B.



Sequencing reactions of ADD-MV passaged once in MRC-5 cells. A: Cycle sequencing reaction using the MMO2a primer. Bases 144, 146, 149, 152 and 153 can not be determined from this reaction. The reaction was carried out using the manufacturer's protocol (II.8.1.2). A 2.5 µl aliquot of the reaction was electrophoresed on a polyacrylamide gel (II.4.2). B: Automated sequencing reaction using the MMO2 primer. The previously undetermined bases were resolved. A gel purified DNA and primer were sent to the automated sequencer in a final volume of 10.5 µl (II.8.1.1).

III.2.3.2 The Sequence of the 5'-end 750 Bases of the ADD-MV M Gene

Before sequence comparisons between low and high passaged virus could be carried out, any M gene sequence differences between virus passaged only once needed to be determined. If the M gene sequence did diverge then a consensus sequence for the 5'-end 750 bases of the M gene of ADD-MV would have had to be determined. The M gene of ADD-MV passaged once in all 4 cell types, namely, MRC-5, Vero, IMR-32 and SK-N-SH cells was sequenced at least twice to ensure any base changes observed were not the result of sequencing errors. The resulting M gene sequence from each of the passaged viruses was identical. When comparing the sequence obtained with that of EdM (III.2.2), two base changes were observed. One was at nucleotide position 222 (Figure 27). This is a cytosine to uracil (C-U) change, which induces a predicted amino acid change from proline to serine. The second base change was at nucleotide position 457 (Figure 28). This was a guanine to adenosine (G-A) mutation, producing a serine to asparagine amino acid change. (Table 10).

Table 10. Base changes observed in passage one of ADD-MV

Cell Line	Nucleotide Position	Mutation	Coding Change
SK-N-SH	222	C - U	Pro - Ser
	457	G - A	Ser - Asn
IMR-32	222	C - U	Pro - Ser
	457	G - A	Ser - Asn
MRC-5	222	C - U	Pro - Ser
	457	G - A	Ser - Asn
VERO	222	C - U	Pro - Ser
	457	G - A	Ser - Asn

The 5'-end 743 base region of the M gene of ADD-MV passaged in each of the four cell lines once was determined by Taq polymerase cycle sequencing (II.8.1.2) and 373A automated sequencing (II.8.1.1). The table shows the changes observed between the published M gene sequence and the M gene sequence of ADD-MV.

III.2.3.3 Sequence Comparison of ADD-MV Passaged 1 and 10 Times

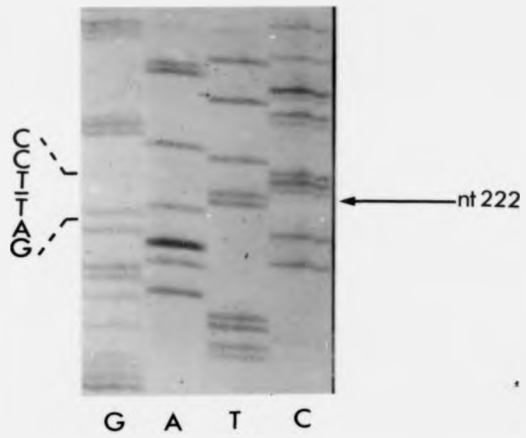
The sequence data obtained from the M gene of ADD-MV passaged 10 times in each of the four cell lines was compared to that determine from passage one (II.2.3.2). The M gene of ADD-MV passaged 10 times in IMR-32 cells and Vero cells did not show any nucleotide changes, however, virus passaged 10 times in SK-N-SH cells contained one change. This occurred in the coding region at nucleotide position 222. A uracil to cytosine change producing a serine to proline substitution, which restores the sequence to that of EdM (III.2.2). Virus passaged in MRC-5 cells exhibited two base changes at passage 10. The first change was at nucleotide position 215. This was a cytosine to guanine change producing a serine to arginine coding change. The second base change was at position 222, a uracil to cytosine change causing a serine to proline coding change (Table 11), which was identical to that change observed in SK-N-SH cells and returned the sequence to EdM (III.2.2).

Table 11. Base changes observed in the M gene of ADD-MV passaged 10 times

Cell Type	Nucleotide Position	Mutation	Coding Change
SK-N-SH	222	U - C	Ser - Pro
MRC-5	215	C - G	Ser - Arg
	222	U - C	Ser - Pro

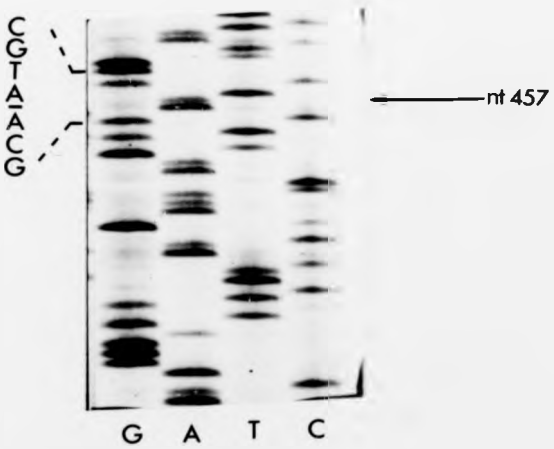
The 743 base region at the 5'-end of the M gene of ADD-MV passaged in all four cell lines 10 times was compared with that determined from virus passaged once (III.2.3.2). The table shows the changes observed between passage 10 and passage one. The sequence was determined by Taq polymerase cycle sequencing (II.8.1.2) and 373A automated sequencing (II.8.1.1).

Figure 27. Nucleotide sequence of ADD-MV M gene showing the C-U base change at position 222



MMO2a cycle sequencing reaction of ADD-MV passaged once in SK-N-SH cells. The reaction was carried out according to the manufacturer's protocol (II.8.1.2). A 2.5 µl aliquot of the reaction was electrophoresed on a polyacrylamide gel (II.4.2).

Figure 28. Nucleotide sequence of ADD-MV M gene showing the G-A base change at position 457



MMO3a cycle sequencing reaction of ADD-MV passaged 10 times in IMR-32 cells. The reaction was carried out in accordance with the manufacturer's protocol (II.8.1.2). A 2.5µl aliquot of the reaction was electrophoresed on a polyacrylamide gel (II.4.2).

III.2.3.4 Sequence of ADD-MV Passaged Once and 10 Times: Conclusions

The M gene of the ADD-MV stock used to infect the four cell lines used in this experiment, differed by two base changes (III.2.3.2) from EdM (III.2.2). Comparison of the sequence of the M gene of ADD-MV after 10 passages in either SK-N-SH or MRC-5 cells revealed a single nucleotide change at nucleotide position 222; reversion of a uracil to a cytosine residue, producing a coding change of serine to proline. The passage history of the ADD-MV used for these experiments is short amounting to a total of only 57 passages in human diploid cells from the original clinical isolate. The passage history of the Edmonston strain of measles virus used in the study carried out by Bellini *et al.* (1986) is unknown. What is known is that the measles virus used in Bellini's study (1986) was propagated in Vero cells, not human diploid cells, although the number of passages in this cell line was not stated. It is possible that the cytosine at position 222, and the loss of the serine codon, may be a consequence of the passage history of the virus subsequent to propagation in human diploid cells or perhaps to the number of passages *in-vitro*.

The aim of this experiment was to determine whether biased hypermutation occurred as a consequence of virus passage in neuroblastoma cells. The data obtained indicates that biased hypermutation did not occur during the propagation of ADD-MV in neuroblastoma cells.

Why the PCR reaction using the MMO1/MMO2 primer pair resulted in the amplification of an 800 base fragment after originally producing the expected size of 1379 bases has not

been fully resolved. This change did occur in conjunction with a change from using AMV-RT to Superscript II to synthesize cDNA, however, there was no corresponding change in the ability of the RSM1/RSM2 primers to amplify the 900 base fragment of huRS virus. Complementary DNA synthesised by both enzymes resulted in the amplification of both 1379 and 800 base fragments (Figure 20), therefore, it is unlikely that the Superscript II enzyme was the sole cause for the change in the PCR product. It would have been necessary to carry out more robust investigation of the PCR cycles used i.e., changes in the annealing temperature and the duration of time spent at that temperature; changes in the extension time . However, the 800 base fragment appeared to contain the 5'-end sequence required for sequence analysis. As this was the main requirement of the PCR product the reduced size of the fragment was not considered detrimental to the project.

III.2.4 Cloning and Sequencing of ADD-MV Passaged 9 Times in IMR-32 Cells

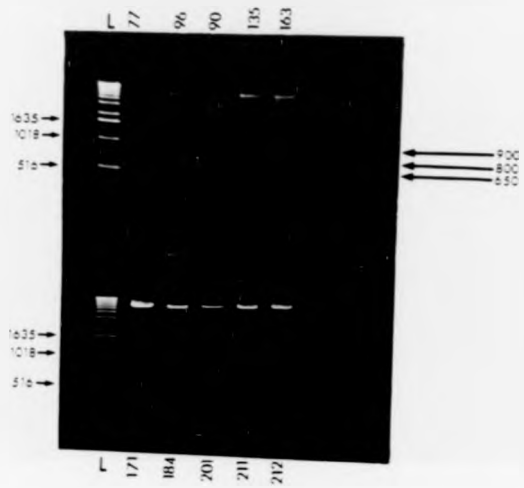
The products of PCR reactions are heterogeneous in nature. Sequencing the PCR product using the chain termination cycle sequencing method results in the sequence of the most abundant molecule in the population being obtained. It is possible that M gene sub-populations may be present, but only at low levels which would not be detected by cycle sequencing. To explore this possibility, the M gene of ADD-MV passaged 9 times in IMR-32 cells was cloned and sequenced. A high passage number in neuroblastoma cells was examined because if any mutational events had occurred, they would be expected to be present at a greater frequency in virus with a longer passage history. Virus passaged in

IMR-32 cells was used because Wong *et al.* (1989) found that Yamagata-1 (SSPE) virus was vulnerable to hypermutation events when passaged in this cell type. Passage 9 was used because virus passaged 10 times in IMR-32 cells had been exhausted.

III.2.4.1 Cloning into the Bacteriophage M13

Total RNA was isolated from ADD-MV material passaged 9 times in IMR-32 cells. This material was reverse transcribed using Superscript II and oligo dT, synthesizing cDNA from the mRNA present. The M gene was amplified using the MMO1/MMO2 primer pair, as described previously (II.3.3.1). As before, the full length of the M gene could not be obtained, only a fragment of approximately 800 bases was amplified. This fragment was cloned into the bacteriophage M13mp18 using the ER cleavage sites inserted at the ends of the fragment during amplification, namely BamHI and XhoI. The BamHI restriction site was present in the multiple cloning site of M13mp18, however, XhoI was not, therefore, the vector was digested with BamHI and Sal I. Sal I digest results in comparable 5' ends as XhoI digests, therefore cloning could proceed. To determine whether clones did contain the M gene insert, the dsDNA replicative form of M13 was digested with Pvu II. This enzyme digests the vector approximately 100 bases either side i.e., 3' and 5', to the multiple cloning site. Positive clones would be expected to contain an insert of approximately 1 kb in size after digestion with Pvu II. Single-stranded DNA was extracted from positive clones and then sequenced. Figure 29 shows the positive M13 clones of the M gene of ADD-MV passaged 9 times in IMR-32 cells.

Figure 29. M gene clones from ADD-MV passaged 9 times in IMR-32 cells



L: 1 kb ladder; numbers 90-212: clone identification numbers. M gene fragments amplified using the MMO1/MMO2 primers were cloned into M13mp18. Potential clones were digested with Pvu II, this enzyme digests M13 approx. 100 bases 5' and 3' of the MCS, therefore, the actual size of the clones are approx. 200 bp smaller than indicated by figure 29. The variation in size of the clones on the upper panel are discussed further (III.2.4.3).

III.2.4.2 Single-Stranded Sequencing of the M Gene Fragments Cloned into M13

Eighty-one M gene clones from ADD-MV passaged 9 times in IMR-32 cells, were sequenced to determine if biased hypermutation was occurring at such a low frequency that it escaped detection by sequencing the PCR product. The clones were sequenced

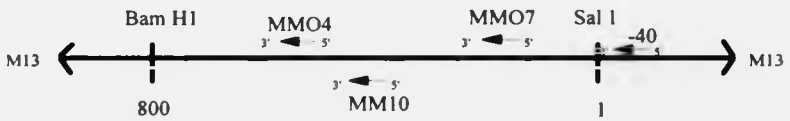
using the -40 primer supplied with the US Biochemicals Sequenase v2.0 sequencing kit (III.2.4.2) and three internal primers (Table 12). EdM (III.2.2) was used to design the internal sequencing primers, which were positioned within the gene so that sequencing data from the most downstream primer would run through the next upstream primer. The position of the sequencing primers within the M gene can be seen in Figure 30.

Table 12. Sequencing primers for single-stranded DNA sequencing

Name	Position (nt)	Sequence	Tm° C
-40	-40 nt into vector	5' CAG CAC TGA CCC TTT TG 3'	52
MMO4	552 to 567	5' CCT AGA AGA ATG CTG G 3'	48
MMO7	134 to 151	5' GGT CAG AGT CAT AGA TCC 3'	54
MM10	427 to 442	5' GGA GTG TCT TCA ACG C 3'	50

The -40 primer was provided with the Sequenase sequencing kit (USB) and primes 40 bases downstream from the MCS of M13. The internal primers were designed using the published M gene sequence for the Edmonston strain of measles virus (Bellini, *et al.*, 1986).

Figure 30. Position of the sequencing primers for single-stranded DNA sequencing in M13mp18



The primers were positioned so that sequence data from the most upstream primer ran through the next downstream primer. Approximately 200-250 bases can be determined from a sequencing reaction electrophoresed for 1.5-2 hrs. Therefore, the sequence reactions from primers MMO7 and MMO4 had to undergo electrophoresis for both 1.5-2 hrs and 6-8 hrs.

III.2.4.3 Sequence of the M Gene Clones of ADD-MV Passage 9 Times in IMR-32 Cells

A method for quickly determining whether any hypermutational events had occurred in the clones is to sequence using a single termination mix. By definition in SSPE viruses, biased hypermutation is a uracil (U) to cytosine (C) change, therefore, either C or T-track sequencing would indicate whether any uracil or cytosine residues had been mutated. This approach was investigated using the cytosine termination mix only, however, very rarely did every C-track reaction produce sequence so comparison was difficult. In those clones where sequence was observed, the resulting C-tracks were comparable when using the -40 primer, but when the internal M gene primers were used unaligned sequence was observed. It was therefore difficult to compare clones. It would have been possible to initially screen the clones using the C-track and the -40 primer to determine whether any base changes had occurred within the 5'-end 200-250 bases, however, out of 41 reactions only half worked well enough for comparison at any one time. It would have been necessary for the reactions to have been repeated, probably several times, before the C-track of all the generated clones had been determined. It proved quicker, and less wasteful of ^{35} S-dATP label, to sequence each clone using the -40 primer in full i.e., all 4 termination mixes were used. Nine times out of 10 each reaction worked well and did not, therefore, have to be repeated. A total of 81 M13 mp18 clones of the M gene of ADD-MV passage 9 times in IMR-32 cell were sequenced with the -40 primer providing approximately 200 bases of sequence in a 1.5-2 hr electrophoresis run. Within this region of the M gene of Yamagata-1 (SSPE) virus Wong *et al.* (1989) observed a total of 19 base changes, thus, the frequency of mutation was approximately 1 in 10. It was presumed, therefore, that if any hypermutational events had occurred during passage of the ADD-

MV there would be changes within the first 200 bases. The results of the initial sequencing reactions determined whether the clones were either, (i) ADD-MV-like i.e. the sequence contained no base changes when compared to that determined from virus passaged once in all four cell types (III.2.3.2), (ii) mutated ADD-MV, i.e. the sequence contained a few base changes when compared to the sequence of virus passaged once, or (iii) the clones were not derived from the ADD-MV M gene. Twenty-one of the 81 clones (25.9%) did not resemble the measles virus M gene and were presumed to be the result of mis-priming or some other artefact of the amplification and cloning procedures. Of the remaining 60 clones, 39 (65%) were identical to ADD-MV. The remaining 21 (35%) contained one or two mutations when compared to the sequence for the M gene of ADD-MV passaged once in all four cell lines. All 21 clones which contained base changes within the first 200 bases of the M gene were sequenced in full. Three of the clones which produced 200 bases of sequence identical to that of the ADD-MV M gene from virus passaged once were also sequenced in full for comparison.

As the full length of the M gene could not be amplified, it was suspected that either a large portion of the M gene had been deleted or that the MMO1 primer had mis-primed during amplification. The sequencing results suggest that a deletion in the M gene was, in fact, responsible for the smaller fragment. The size of the deletions, found in all 24 clones that were sequenced in full, i.e., the 3 clones which did not and the 21 clones which did contain mutations, ranged from 1008 bases to 622 bases. Thirteen of the 21 mutated M genes also contained a 50 base insertion at the 3' end (Figure 31). Each insertion occurred at the same site and contained the same sequence. However, the additional base changes throughout the remainder of the gene varied. Eight of the 13

clones that contained the inserted sequence also contained 4 additional base changes which were identical. The remaining 5 clones containing the insertion also had one or two of the additional changes, but not all four. Table 13 and Figure 32 summarises these results.

III.2.4.3 ADD-MV Passaged 9 Times in IMR-32 Cells: Conclusions

When comparing the sequence obtained for each of the clones of ADD-MV M gene passaged 9 times in IMR-32 (neuroblastoma) cells to the M gene sequence of ADD-MV passaged once (III.2.3.2) a total of 21 additional base changes was observed. It should be noted that only a maximum of 5 of these base changes was seen in any one clone. Of the 21 changes seen, 76% were transitions, the remaining 24% being transversions. Although more mutations were observed in the cloned material than the M gene sequence of ADD-MV passaged once, determined by sequencing the PCR product, there was still no evidence that biased hypermutation had occurred as a consequence of propagation in the neuroblastoma cell line, IMR-32.

The cloning and sequencing results of ADD-MV passaged 9 times in IMR-32 cells indicates that this material contained a high proportion of virus with defective M mRNA. None of the translation initiation codons (AUG) were mutated in the 24 clones sequenced. It is possible, therefore, that the M gene of these clones could be translated. The extent of the translatable open reading frames of the clones varied. The coding region for the 13

Table 13. Summary of the sequencing results of ADD-MV passaged 9 times in IMR-32 cells

Clone	Fragment Size	Deletion	Base Changes	Insertion	Single Base Deletions
95 **	396	310-1318		-	-
96**	626	627-1404		-	-
232 **	752	628-1279	1302 C-U	-	-
77	476	407-1335	98 A-U	-	-
90	446	398-1356	211 A-G	-	-
133	753	715-1366	108 U-A 595 U-C	-	-
135	482	421-1343	199 U-A	-	-
163	646	647-1404	255 U-C	-	-
171 173 178 186 192 196 199 201	782	671-1348	169 A-G 364 U-A 1359 C-U 1367 A-C	50 BASES	1361 C
174	782	671-1348	169 A-G 364 U-A 360 C-U 1367 A-C	50 BASES	1361 C
181	782	671-1348	309 G-A 364 U-A 1359 C-U 1367 A-C	50 BASES	1361 C
184	730	637-1311	158 A-G 303 C-U	-	-

Continuation of Table 13

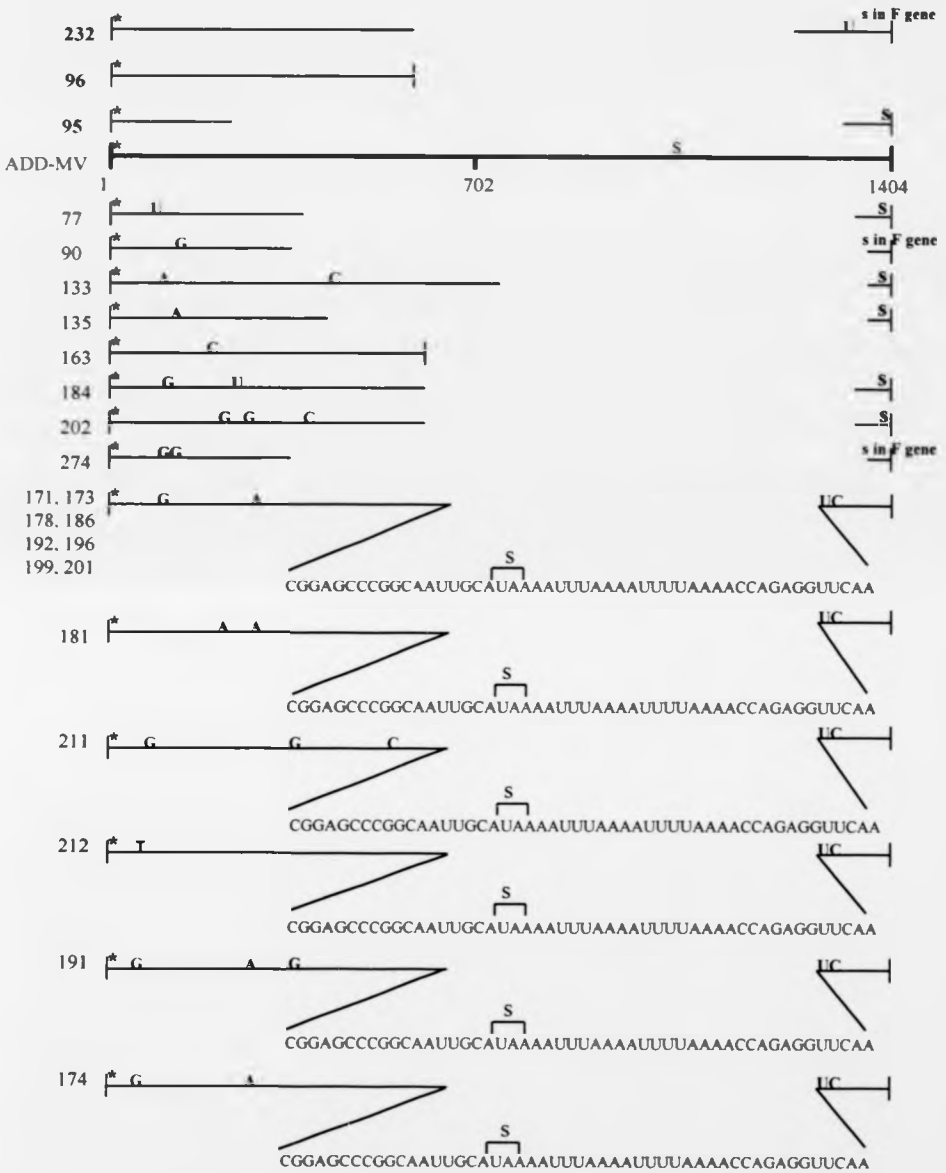
Clone	Fragment Size	Deletion	Base Changes	Insertion	Single Base Deletions
191	782	671-1348	169 A-G 364 U-A 609 A-G 1359 C-U 1367 A-C	50 BASES	1361 C
202	730	637-1311	289 A-G 336 A-G 451 U-C	-	-
211	782	671-1348	169 A-G 466 A-G 604 U-C 1359 C-U 1367 A-C	50 BASES	1361 C
212	782	671-1348	107 C-U 1359 C-U 1367 A-C	50 BASES	1361 C
274	445	398-1357	169 A-G 211 A-G	-	-

The 24 clones sequenced in full are represented in this table. The clones were sequenced using the Sequenase kit (USB) (II.8.2), -40 primer provided with the kit and internal M gene primers (Table13). ** indicate clones in which no mutations were detected within the first 200 nucleotides when compared with the M gene sequence of ADD-MV passaged once in four cell lines (III.2.3.2).

Figure 31. Sequence of the 50 base insertion

Clone 171 was sequenced using the Sequenase kit and the MMO4 primer. A 2.5 µl aliquot of the reaction was electrophoresed for 1.5-2 hrs. Figure 31 shows the sequence of the inserted sequence determined in this clone and 12 other clones.

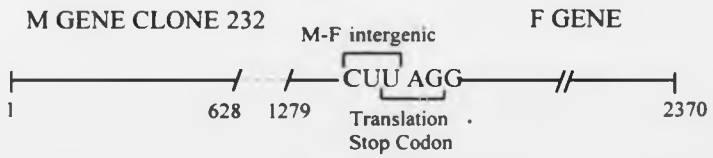
Figure 32. Clones of the M gene of ADD-MV passaged 9 times in IMR-32 cells represented diagrammatically



The 24 clones sequenced in full are represented diagrammatically. The clones were sequenced using the USB Sequenase kit, the -40 primer provided with the kit and internal M gene primers. **232, 96 & 95:** clones which were identical to ADD-MV (III.2.3.2) when the first 200 bp were sequenced initially. *: translation initiation codon; **S:** translation stop codon. **s in F gene:** ORF of these clones continues through the intergenic region to the F gene.

clones containing the 50 base insertion continued for 6 amino acids into the insertion and then terminated by a UAA stop codon (Figure 32). The 6 inserted amino acids were arginine, serine, proline, alanine, isoleucine and alanine. This leaves a 146 base pair untranslated region at the 3' end of the clones. Six clones (77, 95, 133, 135, 184 and 202) which did not contain the 50 base insertion but did have a large region of the M gene deleted, also maintained in-frame stop codons. For these clones the translatable region continued up to a UGA termination codon at nucleotides 1403-1405, leaving only 59 nucleotides untranslated at the 3' end of the gene. The normal stop codon in the M gene of measles virus is a UAG codon at nucleotide position 1038-1041. Clones 163 and 96 contained only the 5' 650 nucleotides of the M gene. Although the translation initiation codon was not destroyed, it is unlikely that these clones could be translated as the M gene sequence is truncated in mid-codon. It is possible, however, that the coding region for these clones could continue through the M-F intergenic region into the 5'-end of the F gene, so producing a novel protein. Clone 232 can, potentially, be translated even though a large proportion of the gene is deleted; however, the stop codon for this clone would be in the transcription start site of the F gene (Figure 33).

Figure 33. Position of the stop codon for clone 232



Clone 232 was generated from PCR using MMO1/MMO2 primers (II.3.3.1). The 750-800 base fragment was cloned directly into M13 using BamHI, XhoI and Sal I digests (II.5.4). Sequencing results showed a 651 base deletion had occurred within the 3'-end of the clone. The ORF was maintained, terminating within the M-F intergenic region.

For clones 274 and 90, again the mRNA could possibly be translated even though a large region of the gene in each clone is deleted; however, the translation stop codon would be at position 585-587 (UGA) in the coding region of the F gene. Thus, the M gene for these clones would continue to be translated through the intergenic region of the M and F gene, and the 5' untranslated 573 nucleotide region of the F gene, assuming that the mRNA is bicistronic. An additional 191 amino acids would be translated from the F gene 5' non-coding region (Figure 34).

The insertion of 50 bases observed in 13 of the clones could be evidence of recombination of measles virus during replication. The inserted sequence was screened in the Blast database, and was found to have 86% identity with human and bovine mitochondrial Leu transfer RNA ($tRNA^{Leu}$). It is possible, therefore, that non-homologous recombination has occurred between mitochondrial RNA and the measles virus M gene during the measles virus replication.

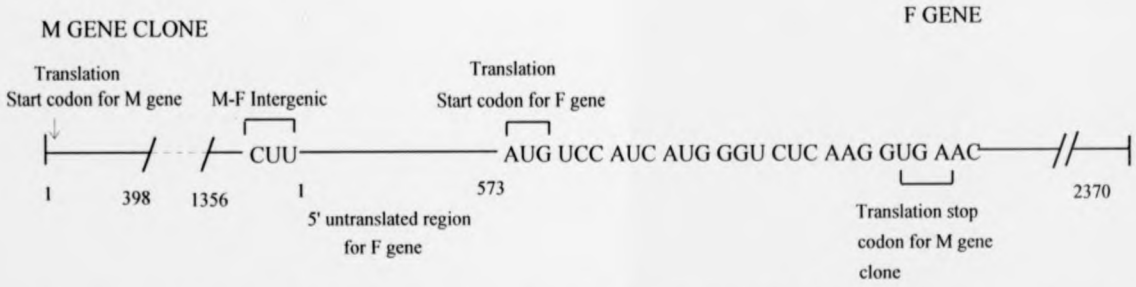
There does not appear to be any sequence homology between the 5' or the 3' end of the inserted sequence and that of EdM (III.2.2). The sequence abruptly changes from measles virus to the inserted sequence after position 672 in the M gene, and from the inserted sequence to EdM at nucleotide position 1348. This would be expected from a cross-over event associated with non-homologous recombination, however, if the inserted sequence was the result of a PCR artefact no homologous region would be observed either. The possibility of recombination verses PCR artefact will be considered further in the discussion (III.2.5).

It should be noted that in the clones which contained it, the inserted sequence had the same orientation and position in each clone. As these clones were generated from PCR the large number of clones containing the insert was probably a consequence of the amplification of the same cDNA species. The slight variation in the number of base changes in the regions flanking the inserts probably reflects polymerase errors that occurred during the amplification stage.

The PCR product cloned was 750-800 bases in size. However, some of the resulting clones (95, 96, 77, 90, 135, 163 & 274) contained fragments of 396-646 bases only (Table 13 & Figure 32). The simplest explanation for this is that the amplified M gene contained base changes which resulted in the formation of the ER sites which were used for the cloning reaction i.e., BamHI and/or XhoI. If two such changes occurred the result could be a large deletion. The primer sequence for MMO1 could be determined from all clones except 96 and 163. It is possible that in these clones only one additional ER site was formed (at position 626 for clone 69, and position 646 for clone 163), therefore, the cloned product would be missing the entire 3 region of the M gene, including the MMO1 primer sequence.

The remaining clones appeared to be 750-800 bases in length, determined by digestion with Pvu II (Figure 29) and yielded sequence of approximately 730 bases (excluding the inserted sequence). Again, the MMO1 primer sequence was observed, therefore, it is unlikely that this primer was mis-priming, and so the amplification of the smaller fragment was due to the presence of deleted M gene species within the virus preparation used for these experiments.

Figure 34. Position of the stop codon for clones 90 and 274



Clones 90 and 274 were generated by amplification of the M gene using MMO1/MMO2 primers (II.3.3.1). The 750-800 base fragment was cloned directly into M13 using BamHI, XhoI and Sal I digests (II.5.4). However, the clones produced were only 446 bases rather than 750-800 bases in size. It is presumed that base changes within the amplified fragments have produced restriction sites that were used in the cloning procedure, resulting in a 958 base deletion at the 3'-end. The ORF remained intact, however, the next available stop codon would not occur until position 596-598 in the coding region of the F gene.

III.2.5 Discussion

III.2.5.1 ADD-MV Passage Experiment

Biased hypermutation is hypothesised to occur as a consequence of the development of a persistent infection in the CNS by measles virus. The aim of the passage of ADD-MV in the neuroblastoma cell lines and the two control cell lines was to develop an *in-vitro* system which would induce hypermutational events. If biased hypermutation is associated with a change in phenotype one could hypothesise that the titre would fall as the degree of hypermutational events had increased. The titre of ADD-MV passaged in each of the 4 cell lines was determined in order to correlated any fall in virus titre with the identification of biased hypermutations. The passage experiments have shown an overall fall in titre from $11.6x10^5$ pfu/ml at passage 4, to $0.19x10^5$ pfu/ml at passage 10 in ADD-MV passaged in Vero cells. This may be indicative of a change in phenotype from a lytic to a persistent phenotype. Rima *et al* (1977) demonstrated that after only 5 passages of undiluted Edmonston B strain of measles virus in Vero cells, the cells had become persistently infected with the virus, therefore, 10 passages of 1:100 diluted virus may be sufficient to initiate a persistent infection. A change in the morphology of the plaques occurs when a persistent infection develops i.e., from large spreading plaque, to a small strand-forming type of c.p.e (Oddo, *et al*, 1961), this change in morphology was not observed in the Vero cells infected with ADD-MV. Persistent measles virus has been shown to produce a low infectivity titre, but maintain a high haemagglutinin titre (Rima *et al*, 1977). Unfortunately, the HA titre of ADD-MV from passage 10 in Vero cells was not determined. The sequence data comparing the M gene of ADD-MV passaged 10 times with that determined from virus passaged only once did not reveal any biased

hypermutations (III.2.3.3), therefore, there is no unequivocal evidence of a transition to a persistent mode of infection of ADD-MV propagated in Vero cells. The virus titres in the remaining cell lines did not reveal an overall fall in titre, thus, there is no evidence of the development of a persistent phenotype in virus passaged in any of the cell lines used.

The method of virus propagation and harvesting i.e., mechanically destroying the monolayer, would possibly select for lytic virus as any cell associated virus would be destroyed along with the cells, thus arguing against the development of a persistent infection. To determine whether this was indeed occurring cell dispersal mechanisms which destroy the monolayer should be compared with enzymatic dispersal methods i.e., EDTA/trypsin. Virus, harvested using each method, would be used to infect sub-confluent cell monolayers at an MOI of <1. Cells would be passaged upto 5 times, depending on the percentage of cells surviving. Those cells which survived would be used in immunofluorescence experiments to determine whether measles virus antigen was present and, thus, whether measles virus persistence had occurred.

The titres determined from virus passage in each of the cell lines used do fluctuate. The most obvious reason for this would be pipetting errors and/or counting errors that were incurred during the plaque assay procedure. Due to the design of the experiment different concentrations of virus were used for each passage as an arbitrary dilution, rather than a standard MOI was used as the inoculum. If a constant MOI had been used any increase or fall in titre could then be attributed to the growth characteristics of the virus in each of the cell lines, rather than the possible increase in DI concentration.

To demonstrate whether DI particles are present in the passaged virus the ability of the passaged virus to reduce virus yield in a mixed infection with standard virus would need to be determined. One millilitre of passaged virus would be mixed with 1 ml of standard virus (the concentration of each virus stock must be known) and used to infect a cell monolayer. As a control 2 ml of the standard virus alone would be used as the inoculum. The titre from each infection would be carried out 3-5 days post-infection. The influence of DI particles would result in a reduced virus titre when compared to the standard virus. Another approach would be to utilize the fact that the effect of DI particles can be diluted out. If a cell monolayer is infected with undiluted virus and another with diluted virus ($1:10^3$), the titre determined from the diluted virus would be expected to be higher than the undiluted inoculum if DI particles were present in the original stock.

III.2.5.2 ADD-MV Passaged Once and 10 Times

A study carried out by Wong *et al.* (1989) revealed that the passage of an SSPE virus (Yamagata-1) in the neuroblastoma cell line, IMR-32, resulted in the accumulation of additional biased hypermutations which were not observed in virus passaged in cells of non-neural origin. It has been suggested that DRADA is responsible for such hypermutations. It is generally accepted that the initial infective material in the CNS of SSPE and MIBE patients is acute measles virus. If this assumption is correct, then acute measles virus must be a substrate for this enzyme to account for the accumulation of biased hypermutations associated with 'SSPE viruses'. Therefore, the aim of this experiment was to determine whether passage of acute measles virus (ADD-MV) in neuroblastoma cell lines was sufficient to induce biased hypermutational events. The

results of this experiment have shown that propagation of ADD-MV in cell lines of neural origin is not sufficient to induce biased hypermutations *in-vitro*. SSPE develops in the brain, which is a very complex organ. The results of passage of ADD-MV in neuroblastoma cells can not be extrapolated to events that may occur in the brain as the cellular environments are very different. However, it has been shown that measles virus double-stranded RNA is a substrate for DRADA (Ecker *et al.*, 1995). What should be noted here is the requirement of double-stranded RNA as a substrate for DRADA. Double-stranded RNA is not an intermediate in measles virus replication and is only likely to occur as a result of local collapse of mRNA on the negative strand template during synthesis (Bass *et al.*, 1989). Therefore, the development of double-stranded RNA during measles virus replication may occur only rarely. Other factors in the CNS may be involved in increasing the propensity of double-stranded RNA formation during replication which are not present in the *in-vitro* environment associated with the experiment described here. This may explain the lack of biased hypermutation observed in this experiment.

Originally, ADD-MV plaque-purified in MRC-5 cells was passaged in the 4 cell lines and, as for the non-purified virus, 1:100 diluted stock was used for the inoculum. Preliminary sequencing data from this virus passaged once indicated the presence of both of the base changes (C-U at position 222 and G-A at position 457) observed in the non-purified virus. It was, therefore, considered unnecessary to continue with the passage of this virus. In retrospect, it would have been more appropriate to continue the passage of plaque-purified virus and to terminate the passage of non-purified virus. Plaque-purified virus is a more homogeneous population of virus, therefore, any base changes observed in

more frequently passaged virus could be attributed to mutational events, rather than the selection of pre-existing variants in the virus population. However, the method of sequencing was Taq polymerase cycle sequencing which will produce sequence of the most predominant population. Plaque-purification would, in all probability, result in a homogeneous virus which represents the predominant virus, therefore, one might not expect the results using plaque-purified virus to be any different from the heterogeneous population.

Mis-priming as an explanation for the amplification of the 750-800 base M gene fragment is unlikely. The results of the cloning reactions of ADD-MV passaged 9 times in IMR-32 cells, described in section III.2.4.2, have shown that in all but two of the clones (No. 96 & 163, see Table 13) the sequence of the MMO1 primer is at the 3' region of the clone, and is not followed by ADD-MV M gene sequence which would be indicative of a mis-priming event. Some clones were produced with 600-700 bases of the 5'-end region of M gene intact i.e., clones 133, 184 and 202. Thus, it is possible that deletion of the M gene was indeed the reason for the 750-800 base fragment. The sequence from the passaged ADD-MV was 743 bases in total, however, not all of the deleted M gene clones (see III.2.4.2) contained the full 5'-end 743 bases. The amplified fragments used for sequencing were either 1379 bases from MMO1/MMO2 (full length M gene), 750-800 bases from MMO1/MMO2 (deleted M gene, containing variable length of the 5' region of the gene) or 750 bases amplified from the MVM1/oligo dT primer pair. In retrospect, this was not an ideal situation. It would have been sufficient to have sequenced only the 1379 base fragment and once that was not able to be amplified, the MVM1/oligo dT fragment. The position where the oligo dT annealed was determined, therefore, the amplified

product was of a defined region, whereas the length of the sequence from the 5'-end of the deleted M gene was variable.

Why was the 1379 base fragment able to be amplified originally? The change in the fragment size produced did not occur in response to increased passage numbers, but occurred abruptly regardless of passage number. The only change in the methodology of the experiment was the use of the Superscript II enzyme. It is possible that this enzyme, in some way, favoured deleted M gene products during cDNA production. Polymerase chain reaction will amplify the most dominant species present in the cDNA pool, hence the amplification of the 800 base fragment.

III.2.5.3 ADD-MV Passaged 9 Times in IMR-32 Cells

Cloning from the PCR product has the disadvantage that it will not give a true representation of the sub-populations of M gene present within a heterogeneous virus population, as PCR itself will amplify the predominant species. A method which will give a more realistic view of the M gene population would involve the production of cDNA library. M gene clones could be identified by making synthetic M gene probes. Confirmation of positive clones would require sequencing. A disadvantage of this method is that hybridisation techniques will identify many clones, each of which would have to be sequenced to confirm that the clone is the required M gene. Due to the time constraints of the project, this method was not considered practical as both ADD-MV passaged material and huRS virus would have to be treated in the same way.

In total 13 clones were found to contain the 50 base insertion, and in each clone the insertion was in the same position. This was presumed to be a consequence of cloning from the PCR product. The fact that there was some variation in the M gene sequence surrounding the insert could represent polymerase errors during the amplification step or pre-existing variants of the M gene. The inserted sequence was found to have high identity with mitochondrial tRNA, suggesting that non-homologous recombination has taken place. If the base changes did represent pre-existing variants then non-homologous recombination could be occurring in 35% of the M gene population. However, it is possible that the PCR conditions are strongly biased towards amplification of short fragments, therefore, full length M gene sequences would be under-represented and over estimation of the abundance of the inserted form could occur. A cross-over event between mitochondrial tRNA and the M gene of ADD-MV, would require regions of homology at the ends of both the insert and the region of the M gene. Indeed, some degree of homology is seen between the 3' end of the inserted sequence and the M gene. Non-homologous recombination is thought to be responsible for the presence of a modified cellular $tRNA^{Asp}$ at the 5' terminus of naturally-occurring DI RNA of plus strand Sindbis virus (Monroe & Schlefinger, 1983). The possibility that the 50 base insertion identified in these clones is a PCR artefact can not be ruled out. However, the fact that other cellular genes have been observed inserted into other virus genomes e.g., 28S ribosomal RNA in influenza virus (Khatchikian *et al.*, 1989) and ubiquitin in bovine virus diarrhoea virus (BVDV) (Meyers *et al.*, 1991) suggests that recombination may also possible in virus containing a negative sense non-segmented RNA genome.

No intact, full length M gene was detected among the clones characterised, each clone contained a deletion ranging from 622 to 1008 bases. The cloned M gene was, on occasion, smaller than expected from the amplified PCR product. This could have been caused by base changes which may introduce ER sites for the enzymes used during the cloning procedure. However, other clones, when sequenced, were of the expected size (750-800 bases), thus, homogeneous modification of the amplified material had not taken place and a deletion in the M gene is still a possible explanation.

Another possible explanation for the deletion could involve the production of a stem loop structure. If regions of the M gene were homologous it is possible that they could base pair. Such secondary structure within the cDNA would result in the formation of a loop. During amplification the polymerase may not continue along the cDNA loop, but, either miss out the secondary structure completely and continue along the single-stranded cDNA region, or dissociate from the template. As a possible illustration of this mechanism a deletion mutant of the coronavirus mouse hepatitis virus (MHV) N gene (Alb4) has been isolated. This mutant has a deletion of 87 nucleotides at the 3'-end of the coding region, resulting in an in-frame deletion of 29 amino acids (Koetzner *et al.*, 1992). This result is similar to the data present here for measles virus, except that coronavirus, while it has a single-stranded RNA genome, has a positive polarity. The genome is also twice the size of measles virus and has been shown to be very amenable to recombination events, unlike measles virus.

Finally, a mutation (T-A) within the H gene of measles virus isolates from the Coventry area has been reported to create an early translation termination signal in that gene,

providing evidence for natural measles virus variability. The expressed protein is predicted to be truncated by 35 amino acids at the C-terminus, which could result in the loss of a neutralising epitope (Outlaw & Pringle, 1995). Thus, the deleted genes detected in the experiments described here may represent variants of measles virus present within a quasispecies population.

In most of the clones sequenced in full the majority of the 3' region of the M gene is deleted, and yet 22 of the 24 clones maintain ORFs. It is possible, therefore, that functional proteins can be produced. Such 3'-end deletions in the M gene of measles virus have also been isolated from the brain of a deceased SSPE patient (Baczko *et al.*, 1993). In Baczko's study 73 cDNA clones of the M gene were generated, 15 of which were full length, the remaining clones contained 3' deletions. This was presumed to be caused by non-specific priming, but was not investigated further. Baczko *et al.* (1993) identified both wild-type-like and hypermutated M gene sequences. Of the deleted M gene population, 15.25% contained wild-type-like sequence, the remainder had hypermutated M gene sequence.

It may be that these deleted M gene products are derived from defective virions which could act as defective interfering particles. However, the ability to amplify these M gene variants was not confined to more highly passaged virus where the concentration of DI particles would be expected to be greater; virus passaged only once also contained these variants. The virus harvested from IMR-32 cells passaged 9 times was still infectious as it was used as the inoculum for passage 10; and it retained plaque-forming activity, therefore, virus with intact M genes was likely to be present at passage 9; from the

number of clones studied here, the frequency of intact M genes in this virus population can be estimated at 1:60 or below.

III.CHAPTER 3

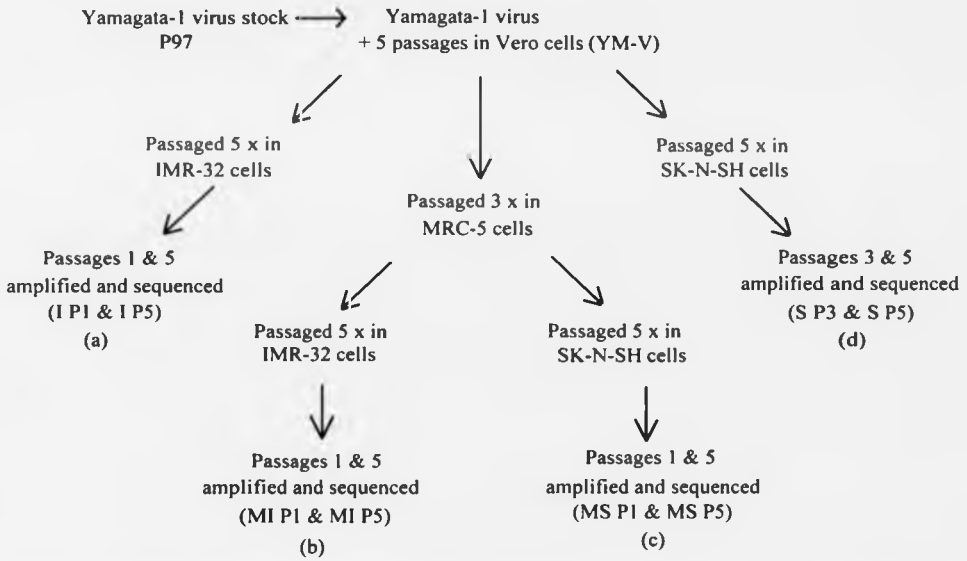
AMPLIFICATION AND SEQUENCING OF THE MATRIX GENE OF THE YAMAGATA-1 (SSPE) VIRUS

Since biased hypermutation was not observed during propagation of ADD-MV in neuroblastoma cells, it is unlikely that a cellular component is responsible for the mutations seen in measles virus genomes isolated from SSPE patients. In order to examine the possible role of the viral genome, virus originally isolated from an SSPE patient was subjected to the same type of analysis.

The Yamagata-1 strain of SSPE virus was kindly provided by Professor M. Homma (Department of Bacteriology, Yamagata University School of Medicine, Yamagata Zao-Iida, Yamagata). It was isolated from brain tissue taken from a 12 year old male patient, who had contracted acute measles virus at the age of 8 months, prior to vaccination. Two portions of the brain material were mechanically treated to disperse the cells, another two portions were treated with EDTA and trypsin. The cell suspensions of the two trypsinised portions and a mechanically treated portion were co-cultivated with uninfected Vero cells in Eagle's minimal essential medium (MEM) supplemented with 10% foetal calf serum (FCS); the second cell suspension derived by mechanical means was centrifuged and the supernatant was used to infect a monolayer of Vero cells. After one day of incubation at 37^{o}C, 1-2 small syncytia were seen in all of the co-cultured cell flasks. Subsequently, the infected cells were passed every 3-6 days by co-cultivation with twice the number of uninfected Vero cells (Homma *et al.*, 1982). The Yamagata-1 virus provided by Professor Homma had been passaged 97 times in Vero cells. This was passaged a further 5 times in Vero cells after receipt and designated YM-V.

In the following experiments two passage protocols (Figure 35) were used. One protocol (Figure 35: branches b & c) was similar to that described by Wong *et al.* (1989). YM-V was passaged three times in human lung fibroblast cells (MRC-5) prior to five passages in neuroblastoma cells (IMR-32 & SK-N-SH). This protocol differs from that described by Wong *et al.* (1989) in that a second neuroblastoma cell line, SK-N-SH cells, was used in addition to IMR-32 cells. Also, the initial Yamagata-1 virus stock had been propagated in Vero cells, then human embryonic lung cells, not MRC-5 cells. The second protocol (Figure 35: branches a & d) involved the passage of YM-V directly in the two neuroblastoma cells (IMR-32 & SK-N-SH) without prior passage in MRC-5 cells. A 25 cm^2 flask of YM-V was dispersed by EDTA/Trypsin (GIBCO BRL) treatment. Half of the infected Vero cells were used to inoculate freshly dispersed neuroblastoma cells or MRC-5 cells. These were then incubated at 37°C to produce passage one. For each passage two 25 cm^2 flasks were inoculated. One flask was harvested by EDTA/Trypsin treatment and used for further passages, the remaining flask was freeze/thawed and aliquoted into 1 ml portions for RNA extraction. Total RNA was extracted after passages 1 and 5 in each cell line and reverse transcribed using Superscript II and oligo dT. The matrix gene was amplified by PCR (III.3.1), cloned into M13 (III.3.2) and sequenced (III.3.3).

Figure 35. Yamagata-1 virus passage protocols



YM-1 virus previously propagated in Vero cells only was used to infect either IMR-32 cells, SK-N-NS or MRC-5 cells as outlined above. All virus was harvested for passage using EDTA/trypsin except IMR-32 cells. The monolayer of these cells could be disrupted by gentle rocking in PBS. Note: Branch (b) mimics the conditions employed by Wong *et al.*, 1989; virus passaged 3 times in SK-N-SH cells was cloned and sequenced instead of passage 1 due to accidental loss of the earlier samples (III.3.5.1).

III.3.1 Polymerase Chain Reaction

Total RNA was extracted from virus passaged once and 5 times in each of the cell lines and mRNA was reverse transcribed using Superscript II and the primer oligo dT. The primers designed to amplify the M gene of ADD-MV did not amplify the M gene of the SSPE virus, presumably as a consequence of a difference in the sequence. Therefore a new set of primers were designed, such that only the first 1000 bases of the M gene mRNA would be amplified. Previous analysis of ADD-MV revealed that the M gene population was heterogeneous with a high proportion of variants which had the 3' region

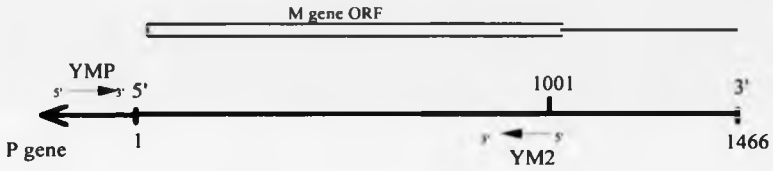
deleted (III.2.4.2). It was possible that the YM-V also contained a high frequency of 3'-end deletions, since Baczko *et al.* (1993) have described M gene variants isolated directly from different regions of the brain of a deceased SSPE patient. However, there have been no reports of 3'-end deleted M gene variants from YM-V, therefore, positioning the YM2 primer 1000 bases downstream from the translation initiation codon of the M gene was not considered an unreasonable approach. The upstream primer (YMP) was located at the 5'-end of the P gene on the assumption that bicistronic mRNA is sufficiently abundant to be reverse transcribed to bicistronic cDNA. This is a hypervariable region of the M gene, where mutational change would be expected and easily detected (III.2.2). Each primer was designed to include unique ER cleavage sites to aid any subsequent cloning procedures. The YMP/YM2 primer pair was used to amplify the M gene from virus passages 1 and 5 in each cell line, for both passage protocols. Using Taq polymerase resulted in the amplification of only faint 1 kilobase (1kb) fragments. To try to increase the concentration of M gene amplified, Vent polymerase was used under the following conditions: 94°Cx45 sec, 56°Cx45 sec and 74°Cx 2 mins for 30 cycles. This did produced a much stronger 1 kb fragment, therefore, this polymerase was used for subsequent reactions. The primers used and the resulting PCR products obtained are outlined below (Table 14; Figures 36 & 37). The minor fragments that have been amplified along with the expected 1 kb fragment may represent non-specific binding of the primers to the template. The very small fragments at the bottom of the gel (< 50 bp) are primer dimers.

Table 14. PCR primers for the M gene of YM-V

Name	Position (nt)	Sequence	ER Sites	Tm °C
YMP	-73 to -54	5' TCT **AGA AGC TTA** CAG CTC AAC TTA CCT GCC 3'	Hind III	60
YM2	1000 to 982	3' CCC GGG **TCG ACA** CGT CGT CGT AAA TGC GG 5'	Sal I	60

The primers were designed using the published sequences of the M and P genes of the Edmonston strain of measles virus (Bellini *et al.*, 1986; Bellini *et al.*, 1985). YMP was position at the 3'-end UTR of the P gene. YM2 was positioned 1000 bases downstream of the translation initiation codon of the M gene. Endonuclease restriction (ER) sites were included in the design of the primers. To ensure efficient cleavage the sites used for cloning were positioned 4-5 bases from the end of the primer sequence.

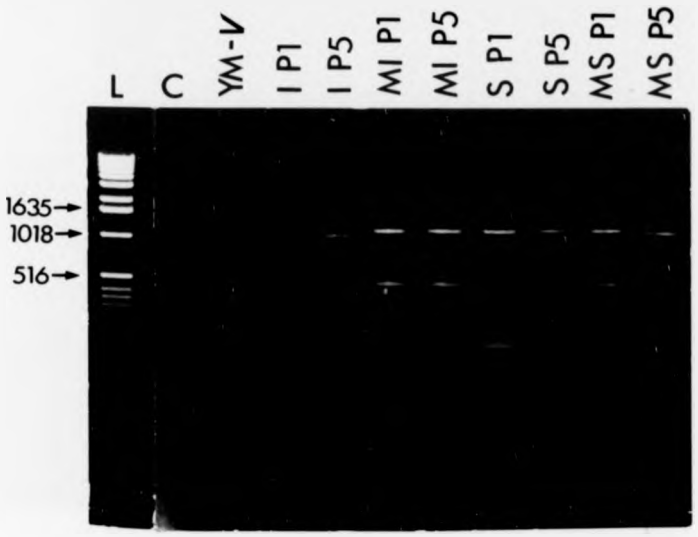
Figure 36. Position of the PCR primers within the M gene of YM-V



The 1000 base fragment amplified covers a hypervariable region of the M gene where mutational change would be expected and easily detected (III.2.2).

The majority of the amplified products are of the predicted size i.e., 1 kb. However, the product from YM-V passaged 5 times in IMR-32 cells and virus passaged in MRC-5 cells followed by 5 passages in SK-N-SH appears to be approximately 50 bases smaller, indicating that either the primers are mis-priming or that the M gene of this passaged virus contained a small deletion. These products were cloned into the bacteriophage M13 and sequenced using the USB Sequenase kit, and were found to be an 'unknown sequence' (Appendix A) which was reproducibly amplified using the YMP/YM2 primer pair presumably as a consequence of mis-priming. Subsequently it was found that virus

Figure 37. YM-V M gene fragments amplified by PCR



L: 1kb ladder; C: negative control; YM-V: initial virus stock; I P1: virus passaged once in IMR-32 cells; I P5: virus passaged 5 times in IMR-32 cells; MI P1: virus propagated in MRC-5 cells, followed by one passage in IMR-32 cells; MI P5: virus propagated in MRC-5 cells, followed by 5 passages in IMR-32 cells; S P3: virus passaged 3 times in SK-N-SH cells; S P5: virus passaged 5 times in SK-N-SH cells; MS P1: virus propagated in MRC-5 cells, followed by once passaged in SK-N-SH cells; MS P5: virus propagated in MRC-5 cells, followed by 5 passages in SK-N-SH cells. The PCR amplification was carried out as previously described (II.3.3.2). Half of the PCR product (50 µl) was electrophoresed on a 1% agarose TAE gel. The 1 kb fragment was excised from the gel and subjected to Geneclean procedures (II.5.1), following the manufacturer's protocol, prior to cloning.

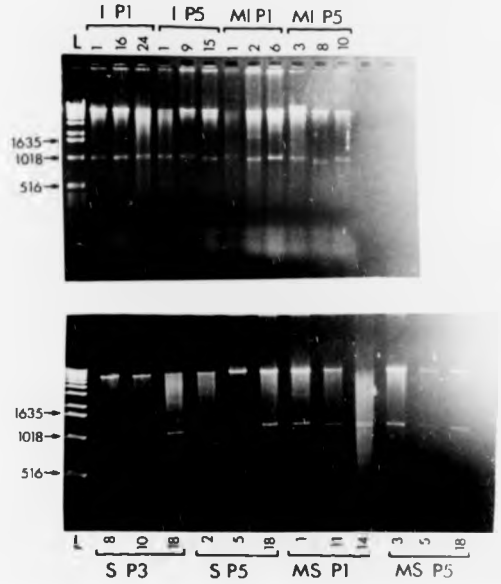
passaged in IMR-32 cells once, and virus passaged in MRC-5 cells followed by 5 passages in IMR-32 cells also yielded this 'unknown sequence', although not resolved as a shorter amplification product. The unknown sequence was screened using the Blast Nucleotide Sequence Database, however, no positive identification was found. This sequence did not have any homology with any nucleotide sequence stored in the database (upto January 1996).

III.3.2 Cloning into the Bacteriophage M13

It was considered necessary to clone the amplified M gene fragments as the cloning of the ADD-MV indicated that the M gene mRNA exists as a heterogeneous population (III.2.4). The resulting M gene fragments were gel purified using the Geneclean II (BIO 101) protocol (II.5.1) and cloned into the bacteriophage M13 (II.5.4).

The M gene was cloned into the bacteriophage M13mp18 using the ER sites inserted at both the 3' and 5' ends of the fragment during amplification, namely Sal I and Hind III. Both of these restriction sites are present in the multiple cloning site of M13mp18 and mp19. Inadvertently, the cloning reactions were carried out using a stock of mp18 that was contaminated with mp19. During the cloning reactions some of PCR products ligated with the mp19 vector. Thus, some of the inserted sequences were in the opposite orientation to those cloned into the mp18 vector. Figure 38 shows positive M13 clones of the M gene of YM-V. Clone 2 of Yamagata-1 virus passaged 5 times in SK-N-SH cells (S P5) is obviously smaller than the expected 1 kb insert. Likewise, clone 15 of virus passaged 5 times in IMR-32 cells (I P5), clone 2 of virus passaged in MRC-5 cells prior to one passaged in IMR-32 cells (MI P1), and clone 8 of virus passaged in MRC-5 cells followed by 5 passages in IMR-32 cells (MI P5) are all slightly smaller than the predicted 1 kb insert. These observations are discussed in further detail elsewhere (III.3.5.3; III3.5.4; III.3.6.1; III.3.6.4). The resulting single-stranded DNA was then sequenced.

Figure 38. Positive M13 clones of the M gene of YM-V



L: 1kb ladder; 24 etc.: clone ID numbers; I P1: clones from virus passaged once in IMR-32 cells; I P5: clones from virus passaged 5 times in IMR-32 cells; MI P1: clones from virus propagated in MRC-5 cells, followed by one passage in IMR-32 cells; MI P5: clones from virus propagated in MRC-5 cells, followed by 5 passages in IMR-32 cells; S P3: clones from virus passaged 3 times in SK-N-SH cells; S P5: clones from virus passaged 5 times in SK-N-SH cells; MS P1: clones from virus propagated in MRC-5 cells, followed by once passaged in SK-N-SH cells; MS P5: clones from virus propagated in MRC-5 cells, followed by 5 passages in SK-N-SH cells. Amplified M gene clones were ligated into M13 vector. Both single-stranded and double-stranded DNA was isolated from overnight cultures of clones. Double-stranded DNA was digested with Sal I and Hind III to determine which clones contained the correct insert (1 kb).

II.3.3 Sequencing the 1 kb Fragment of the M Gene of YM-V

The single-stranded DNA produced from 3 clones derived from passages 1 and 5 in each cell line was sequenced using US Biochemicals Sequenase v2.0 sequencing kit. Five internal sequencing primers were designed in order to sequence the full 1 kb fragment of the M gene, along with the -40 primer supplied with the Sequenase kit. The primers used

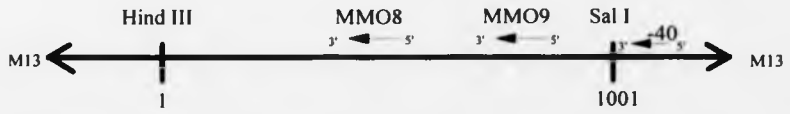
for sequencing and their positions within the M gene can be seen in Table 15, and in Figures 39 & 40, below.

Table 15. Sequencing primers for single-stranded DNA sequencing

Name	Position	Sequence	Tm °C
-40	-40 nt into vector	5' CAG CAC TGA CCC TTT TG 3'	52
MMO4	nt 552 to 567	5' CCT AGA AGA ATG CTG G 3'	48
MMO7	nt 134 to 151	5' GGT CAG AGT CAT AGA TCC 3'	54
MMO8	nt 151 to 134	3' GGA TCT ATG ACT CTG ACC 5'	48
MM09	nt 567 to 552	3' CCA GCA TTC TTC TAG G 5'	54
MM10	nt 427 to 442	5' GGA GTG TCT TCA ACG C 3'	50

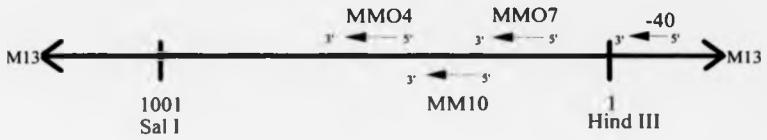
The -40 primer was provided with the Sequenase sequencing kit (USB) and primes 40 bases downstream from the MCS of the vector. The internal primers were designed using the published M gene sequence for the Edmonston strain of measles virus (Bellini *et al.*, 1986). Primer MM08 is complementary to MM07 and MMO9 is complementary to MMO4. These primers are used to sequence the M gene products cloned into M13mp19.

Figure 39. Position of the sequencing primers for single-stranded DNA sequencing in M13 mp19



The primers were positioned so that sequence data from the most upstream primer will run through the next downstream primer. Approximately 200-250 bases can be sequenced when the reactions are electrophoresed for 1.5-2 hrs. In order to sequence the 5' region of M gene products cloned into mp19, reactions from the MMO8 primer had to electrophoresed for both 1.5-2 hrs and 6-8 hrs in order to sequence to the beginning of the M gene.

Figure 40. Position of the sequencing primers for single-stranded DNA sequencing in M13 mp18



The primers were designed so that sequence data from the most upstream primer ran through the next downstream primer. Approximately 200-250 bases can be determined from a reaction electrophoresed for 1.5-2 hrs, therefore, to determine the 3' region of the M gene inserted into mp18, reactions using the MMO4 primer had to be electrophoresed for 1.5-2 hrs and 6-8 hrs.

III.3.4 M Gene 1 kb Fragment Sequence of YM-V

The supernatant from Vero cells infected with YM-V (passage 96) was titred using Vero cells to determine whether any lytic virus was produced. The titre was found to be very low, 980 pfu/ml. This is in accordance with the observations of Homma *et al.*, (1982), who found that less than 1000 pfu of infectious virus could be recovered from the supernatants of freeze/thawed preparations of the cells

The M gene of YM-V virus was amplified, cloned and sequenced initially to confirm the presence of the mutated M gene described previously by Wong *et al.* (1989). Three clones of YM-V were sequenced. Whereas two of the three clones, clones 4 and 7, resembled EdM (III.2.2), one clone, clone 5, resembled the M gene sequence of Yamagata-1 virus described by Wong *et al.* (1989).

Figure 41. YM-V M gene sequence data from clones 4, 5 & 7

```
EdM    AGGAGCAAAGUGAUUGCCUCCCAAGUUCCACAAUGACAGAGAUCUACGACUUCGACAAGU  60
4      ............................................................
7      ............................................................
5      ........................C.......C...........C.....C.........
YM-1   ........................C.......C...........C.....C.........

EdM    CGGCAUGGGACAUCAAAGGGUCGAUCGCUCCGAUACAACCCACCACCUACAGUGAUGGCA  120
4      U...........................................................
7      ............................................................
5      ................G.....................G.........C..........G.
YM-1   ................G.....................G.........C..........G.

EdM    GGCUGGUGCCCCAGGUCAGAGUCAUAGAUCCUGGUCUAGGCGACAGGAAGGAUGAAUGCU  180
4      ............................................................
7      ............................................................
5      ............................................................
YM-1   ..........................................................C......C

EdM    UUAUGUACAUGUUUCUGCUGGGGGUUGUUGAGGACAGCGAUCCCCUAGGGCCUCCAAUCG  240
4      ..........................................U.................
7      ..........................................U.................
5      ............................................................
YM-1   .C..........................................................

EdM    GGCGAGCAUUUGGGUCCCUGCCCUUAGGUGUUGGCAGAUCCACAGCAAAGCCCGAAAAAC  300
4      ............................................................
7      ............................................................
5      ..................................................A......G...
YM-1   ..................................................A......G...

EdM    UCCUCAAAGAGGCCACUGAGCUUGACAUAGUUGUUAGAGCUACAGCAGGGCUCAAUGAAA  360
4      ............................................................
7      ............................................................
5      ......................G..A.............................U......
YM-1   ......................G..A.....................................

EdM    AACUGGUGUCUACAACAACACCCCACUAACUCUCCUCACACCUUGGAGAAAGGUCCUAA  420
4      ............................................................
7      ............................................................
5      ..........................................................C..
YM-1   ..........................................................C..

EdM    CAACAGGGAGUGUCUUCAACGCAAACCAAGUGUGCAGUGCGGUUAAUCUGAUACCGCUCG  480
4      ....................................A.......................
7      ....................................A.......................
5      ....................................A.............G.......G.
YM-1   ....................................A.............G.......G.

EdM    AUACCCCGCAGAGGUUCCGUGUUGUUUAUAUGAGCAUCACCCGUCUUUCGGAUAACGGGU  540
4      ............................................................
7      ............................................................
5      .C........................C..C................CC.U...C.......
YM-1   .C........................C..C................CC.U...C.......
```

```
EdM    AUUACACCGUUCCUAGAAGAAUGCUGGAAUUCAGAUCGGUCAAUGCAGUGGCCUUCAACC  600
4      ............................................................
7      ............................................................
5      .C..........C.................G.............................
YM-1   .C..........C.................G.............................

EdM    UGCUGGUGACCCUUAGGAUUGACAAGGCGAUAGGCCCUGGGAAGAUCAUCGACAAUACAG  660
4      ............................................................
7      ............................................................
5      ................A.............U.........................G...
Y      ................A.............U.........................G...

EdM    AGCAACUUCCUGAGGCAACAUUUAUGGUCCACAUCGGGAACUUCAGGAGAAAGAAGAGUG  720
4      ............................................................
7      ............................................................
5      ....................................G.....................
YM-1   ....................................G.....................

EdM    AAGUCUACUCUGCCGAUUAUUGCAAAAUGAAAAUCGAAAAGAUGGGCCUGGUUUUUGCAC  780
4      ............................................................
7      ............................................................
5      ......................G.....................................
YM-1   ......................G.....................................

EdM    UUGGUGGGAUAGGGGGCACCAGUCUUCACAUUAGAAGCACAGGCAAAAUGAGCAAGACUC  840
4      ............................................................
7      ............................................................
5      ............................C...............................
YM-1   ............................C...............................

EdM    UCCAUGCACAACUCGGGUUCAAGAAGACCUUAUGUUACCCGCUGAUGGAUAUCAAUGAAG  900
4      ............................................................
7      ............................................................
5      ............................C.........A.CA..................
YM-1   ............................C.........A.CA..................

EdM    ACCUUAAUCGAUUACUCUGGAGGAGCAGAUGCAAGAUAGUAAGAAUCCAGGCAGUUUUGC  960
4      ............................................................
7      ............................................................
5      ...CC.....................................C.............C...
YM-1   ...CC.....................................C.............C...

EdM    AGCCAUCAGUUCCUCAAGAAUUCCGCAUUUACGACGACGUG                    1001
4      ....................................
7      ....................................
5      ............C......C................
YM-1   ............C......C................
```

EdM corresponds to the published Edmonston measles virus M gene sequence (Bellini *et al*, 1986); 4,7 and 5 correspond to the three clones of the YM-V M gene sequence; YM-1 is the M gene sequence of Yamagata-1 virus propagated in Vero cells only, described by Wong *et al*, 1989. **AUG** represents the translation initiation codon. The 1 kb fragment amplified using the YMP/YM2 primer pair was cloned into M13. Three resulting clones were sequenced using the Sequenase v2.0 sequencing kit (USB).

Figure 41 shows that clones 4 and 7 differ from EdM (III.2.2) at two sites only; a C-U substitution at position 222 and a G-A substitution at position 457. Clone 4 contains an additional nucleotide change at position 61 (C-U) (Table 16).

Table 16. Comparison of YM-V clones 4 & 7 with EdM

Clone 4		Clone 7	
Position	Base Change	Position	Base Change
61	C-U	-	-
222	C-U	222	C-U
457	G-A	457	G-A

The M gene of YM-V was cloned into the bacteriophage M13. The sequence of 3 clones was determined using the Sequenase sequencing kit (USB). Comparison of the sequence from clones 4 and 7 with the published M gene sequence (Bellini *et al.*, 1986) revealed the base changes tabulated above.

Clone 5, however, is highly mutated when compared to EdM. A total of 43 base changes were observed between clone 5 and EdM (III.2.2); 37 were transitions, 24 of which were either U-C or C -U, indicative of biased hypermutation. The remaining 6 base changes were transversions, either C-G or A-U. This clone differs only at positions 173, 180 and 182 from that described by Wong *et al.* (1989); the U-C base changes at these positions are not present. This clone contains an additional base change not observed by Wong *et al.* (1989) at position 354 (A-U) (Table 17). The translation initiation codon of clone 5 is destroyed by U-C transition at position 34, as described by Wong *et al.* (1989).

Table 17. Base differences observed between the M gene of YM-V clone 5, the M gene of Yamagata-1 virus described by Wong *et al.* (1989) and EdM

Nucleotide Position	YM-V Clone 5	Yamagata-1	EdM
26	C	C	U
34	C	C	U
45	C	C	U
51	C	C	U
77	G	G	A
99	G	G	C
108	C	C	U
119	G	G	C
173	U	U	U
180	U	U	U
182	U	U	U
290	A	A	G
297	G	G	A
321	G	G	C
324	A	A	G
354	U	A	A
418	C	C	U
457	A	A	G
471	G	G	A
479	G	G	C
482	C	C	U
506	C	C	U
509	C	C	U
526	C	C	U
527	C	C	U
529	U	U	C
533	C	C	U
542	C	C	U
554	C	C	U
575	G	G	A
617	A	A	G
632	U	U	A
657	G	G	A
699	G	G	A
746	G	G	U
811	C	C	U
871	C	C	U
881	A	A	G
883	C	C	U
884	A	A	G
904	C	C	U

Continuation of Table 17

Nucleotide Position	YM-V Clone 5	Yamagata-1	EdM
905	C	C	U
940	C	C	U
957	C	C	U
974	C	C	U
981	C	C	U

Comparison of the sequence from clone 5 with the M gene sequence of Yamagata-1 virus passaged in Vero cells (Wong *et al.*, 1989) and EdM (Bellini *et al.*, 1989) revealed the base changes tabulated above.

III.3.5 The Sequence of the 1 kb Fragment of YM-V From Early Passage in Neuroblastoma Cells

III.3.5.1 M Gene Sequence of YM-V Passage 3 Times in SK-N-SH Cells

The M gene of YM-V passaged 3 times in the SK-N-SH neuroblastoma cell line was cloned into M13mp19 and sequenced. Originally, virus passaged only once was to be sequenced, however, due to accidental loss of the earlier samples virus passaged 3 times was the earliest passage material available for cloning. Eleven clones were sequenced using the -40 primer which produced approximately 250 bases of sequence, and all were found to be similar to EdM (III.2.2). Three clones (nos. 8,10 and 18) were sequenced in full. All three clones contained two base changes when compared to EdM (III.2.2), namely a C-U nucleotide transition at position 222, and a G-A change at position 457. Clone 18 contained one additional base change at position 225. This was a C-U transition and is a silent mutation. Table 18 shows the sequence differences between the three clones and EdM (III.2.2).

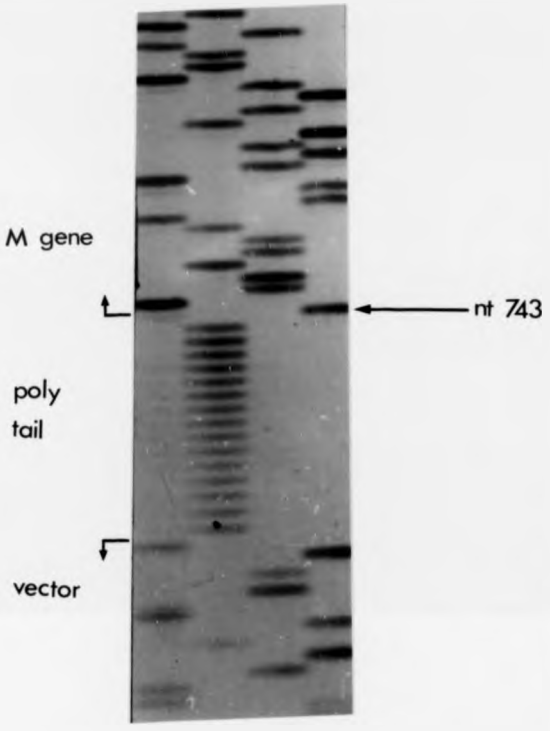
Table 18. Comparison of YM-V passaged 3 times in SK-N-SH cells with EdM

Clone 8		Clone 10		Clone 18	
Position	Base Change	Position	Base Change	Position	Base Change
222	C-U	222	C-U	222	C-U
-	-	-	-	225	C-U
457	G-A	457	G-A	457	G-A

The M gene of YM-V passaged 3 times in SK-N-SH cells was cloned into the bacteriophage M13. The sequence of 3 clones was determined using the Sequenase sequencing kit (USB). Comparison of the sequence of each clone with the published M gene sequence of the Edmonston strain of measles virus (Bellini *et al.*, 1986) revealed the base changes tabulated above.

It should also be noted that the size of the cloned sequence is smaller than expected (Figure 37). The amplified M gene fragment was approximately 1000 bases, however, the inserted sequence was only 743 bases in length. Possible explanations are discussed later (III.3.9). Sequence data from the -40 primer should correspond to the 3'-end if the PCR fragment is cloned into mp19. A 2 hr run of this sequencing reaction produced approximately 250 bases of sequence. A poly A tail of 15 adenosine residues was found prior to the M gene sequence (Figure 42). However, the 15 bases preceding the oligo T tail do not correspond with measles virus M gene sequence after nucleotide position 760. The exact reason for this result has not been fully resolved. However, contamination of the vector stock with ADD-MV M gene is the most likely explanation as ADD-MV amplified using the MVM1/oligo dT primer pair produced sequence of 743 bases in length, due to the priming of oligo dT at positions 743-759 (III.2.2; Figure 21).

Figure 42. The 3'-end sequence of YM-V Passaged 3 Times in SK-N-SH cells



The sequence reaction using the -40 primer provided with the Sequenase sequencing kit. Clone 10 of the M gene of YM-V passaged 3 times in SK-N-SH cells. The poly A tail can be seen preceding the M gene sequence from position 743. A 1 kb fragment of the M gene was amplified using the YMP/YM2 primer pair and subsequently cloned in the bacteriophage M13.

III.3.5.2 M Gene Sequence of YM-V Propagated in MRC-5 Cells Followed by One Passage in SK-N-SH

The M gene of YM-V passage 3 times in MRC-5 then once in the neuroblastoma cell line, SK-N-SH (Figure 35), was cloned into M13. A total of 10 clones were sequenced with the -40 primer. Five of these were found to resemble the M gene of measles virus, one of the remaining clones was the unknown sequence (Appendix A) and the remaining

4 clones were not related to measles virus or the previously isolated unknown sequence, and were presumed to be artefacts produced during the cloning process. Three of the 5 measles virus clones were then sequenced in full (960 bases). The amplified fragment of clone 1 was ligated into M13mp18, while the amplified fragments of clones 11 and 14 were ligated into M13mp19. Clone 1 produced sequence which resembled EdM. (Table 19). The other two clones, 11 and 14, were identical to the M gene sequence of Yamagata-1 virus described by Wong *et al.* (1989), except that three U-C transitions at positions 173, 180 and 182 are not present (Table 20).

Table 19. Comparison of clone 1 from YM-V propagated in MRC-5 cells followed by one passaged in SK-N-SH cells, with EdM

Clone 1	
Position	Base Change
222	C-U
457	G-A

The M gene of YM-V passaged in MRC-5 cells followed by one passage in SK-N-SH cells was cloned into the bacteriophage M13. The sequence of 3 clones was determined using the Sequenase sequencing kit (USB). Comparison of the sequence from clone 1 with the published M gene sequence (Bellini *et al.*, 1986) revealed the base changes tabulated above.

Table 20. Comparison of clones 11 and 14 of YM-V propagated in MRC-5 cells followed by one passaged in SK-N-SH cells with the M gene sequence of Yamagata-1 virus described by Wong *et al.* (1989)

Clone 11		Clone 14	
Position	Base Change	Position	Base Change
173	C-U	173	C-U
180	C-U	180	C-U
182	C-U	182	C-U

The M gene of YM-V passaged in MRC-5 cells followed by one passaged in SK-N-SH cells was cloned into the bacteriophage M13. The sequence of 3 clones was determined using the Sequenase sequencing kit (USB). Comparison of the sequence from clones 11 and 14 with the M gene sequence of Yamagata-1 virus propagated in Vero cells only (Wong *et al.*, 1989) revealed the base changes tabulated above.

III.3.5.3 M Gene Sequence of YM-V Passaged Once in IMR-32 Cells

The M gene of YM-V passaged once in the neuroblastoma cell line IMR-32 was cloned into M13mp18. The intention was to sequence three clones. However, the sequence amplified from this material using the same primers as before (YMP and YM2) was the unknown sequence described in Appendix A.. A total of 25 clones was sequenced from three separate cloning reactions. The PCR fragment was of the expected size i.e., 1 kb, as were the digest products of the clones when screening for positive clones. The PCR reactions were repeated using uninfected cells; no fragment was amplified, therefore, it is unlikely that the cloned sequence is an artefact of cellular origin.

III.3.5.4 M Gene Sequence of YM-V Propagated for One Passage in IMR-32 Cells Preceded by three passages in MRC-5 Cells

The M gene of YM-V passaged 3 times in MRC-5 cells, followed by one passage in IMR-32 cells (Figure 35; branch b) was cloned into M13mp18. Four clones were sequenced with the -40 primer and found to be positive for the M gene of measles virus. Three clones (1,2 and 6) were then sequenced in full (960 bases). All three clones possessed a sequence which was similar to that described by Wong *et al.* (1989). Table 21 summaries the base changes observed when the sequence of the three clones are compared with the M gene sequence of Yamagata-1 virus (Wong *et al.*, 1989).

Table 21. Comparison of clones 1, 2 and 6 of YM-V propagated in MRC-5 cells followed by a single passage in IMR-32 cells with the M gene of Yamagata-1 virus described by Wong *et al.* (1989)

Clone 1		Clone 2		Clone 6	
Position	Base Change	Position	Base Change	Position	Base Change
45	C-U	26	C-U	-	-
51	C-U	-	-	-	-
173	C-U	173	C-U	173	C-U
180	C-U	180	C-U	180	C-U
182	C-U	182	C-U	182	C-U

The M gene of YM-V passaged in MRC-5 cells followed by one passage in IMR-32 cells was cloned into the bacteriophage M13. The sequence of 3 clones was determined using the Sequenase sequencing kit (USB). Comparison of the sequence from clones 1,2 and 3 with the M gene sequence of Yamagata-1 virus passaged in Vero cells only (Wong *et al.*, 1989) revealed the base changes tabulated above.

The 3 clones differed from the M gene sequence described by Wong *et al.* (1989) at positions 173, 180 and 182. Clone 1 does not contain the U-C transitions at positions 45 and 51 which were described by Wong *et al.* (1989); and clone 2 does not contain the U-C transition at position 26, however, all three clones do contain the U-C mutation at position 34 which destroys the translation initiation codon, thus, preventing the translation of M protein.

III.3.6 Sequence of the 1 kb M Gene Fragment of YM-V Passaged 5 Times in the Neuroblastoma Cells

III.3.6.1 M Gene Sequence of YM-V Passaged 5 Times in SK-N-SH Cells

The M gene of YM-V passaged 5 times in SK-N-SH cells was cloned into M13mp18. A total of 19 clones were sequenced using the -40 primer. Fifteen of these clones were

found to be the unknown sequence (Appendix A), the remaining 4 clones resembled the M gene of EdM (III.2.2). Three of these four clones (nos. 2, 5 and 18) were sequenced in full (clones 2 & 5 produced 726 bases of sequence; clone 18 produced 960 bases of sequence). The reduced M gene fragment sequenced from clones 2 and 5 is the result of a base change at position 726, which results in the formation of a Sal I ER site. This enzyme is used in the cloning process, resulting in the truncation of the M gene clone. The sequence determined from each of the 3 clones resembled EdM (III.2.2). Table 22 summaries the base differences between the 3 clones and EdM (III.2.2).

Table 22. Comparison of clones 2, 5 and 18 of YM-V passaged 5 times in SK-N-SH with EdM

Clone 2		Clone 5		Clone 18	
Position	Base Change	Position	Base Change	Position	Base Change
9	A-G	-	-	61	C-U
222	C-U	222	C-U	222	C-U
457	G-A	457	G-A	457	G-A
*726	U-G	*726	U-G	-	-
-	-	-	-	542	U-C
-	-	-	-	543	U-C
-	-	-	-	545	C-A

The M gene of YM-V passaged 5 times in SK-N-SH was cloned into the bacteriophage M13. The sequence of 3 clones was determined using the Sequenase sequencing kit (USB). Comparison of the sequence from clones 2, 5 and 18 with the published M gene sequence (Bellini *et al.*, 1986) revealed the base changes tabulated above. * indicates the position of the base change which introduces a Sal I restriction site. This enzyme was used in the cloning procedure, so explaining the smaller size of the inserted M gene fragment.

III.3.6.2 M Gene Sequence of YM-V Passaged in MRC-5 Cells Followed by 5 Passages in SK-N-SH Cells

The M gene of YM-V passaged three times in MRC-5, then 5 times in the neuroblastoma cells, SK-N-SH (Figure 35; branch c), was cloned into M13mp18. A total of 29 clones, generated from 3 separate cloning reactions, were sequenced with the -40 primer, all of which yielded the unknown sequence describe in Appendix A. The PCR reactions were repeated using uninfected SK-N-SH cells, no fragment was amplified, therefore it seems unlikely that this sequence is an artefact of cellular origin.

III.3.6.3 M Gene Sequence of YM-V Passaged 5 Times in IMR-32 Cells

The M gene of YM-V passaged 5 times in IMR-32 cells was amplified by PCR and cloned in M13mp18. A total of 18 clones, generated in three separated cloning reactions, was sequenced using the -40 primer. The sequence obtained from all of the clones was the unknown sequence described in Appendix A. The PCR reactions were repeated using uninfected IMR-32 cells, again, no fragment was amplified, therefore, it seems unlikely that this sequence is an artefact of cellular origin.

III.3.6.4 M Gene Sequence of YM-V Passaged in MRC-5 Cells Followed by Passage 5 Times in IMR-32 Cells

The M gene of YM-V passaged 3 times in MRC-5 cells, followed by 5 passages in IMR-32 cells was amplified by PCR and cloned into M13mp18. A total of 21 clones, generated in three separated cloning reactions, was sequenced with the -40 primer. Nineteen of the 21 clones were the unknown sequence described in Appendix A. The sequence obtained from one of the remaining two clones showed high identity with several *Homo sapiens* cDNA clones ranging from 66% identity to 73% when searched through the Blast Database. The final clone showed high identity (71%) with the *Haemophilus influenzae* recF, dnaN, dnaA, tbp1-A and tbp2-B genes from bases 1052980 to 1063176 when screened through the Blast database. The primer sequence could not be read from the gel of the clones which have identity with *Homo sapiens* cDNA clones and *H. influenzae* genes which suggests the these sequences are in no way specific to measles virus infections. It is likely that they are simply artefacts of the cloning reactions.

III.3.7 Amplification and Cloning of the M gene of YM-V Passage Material From Which Only Unknown Sequence Had Been Recovered

III.3.7.1 Nested Polymerase Chain Reaction

Nested PCR is a more specific method because two reactions, using different primer pairs, are required to amplify the material in question. The increased specificity was required to determine whether measles virus sequence could be recovered from the YM-V

passaged material where only the unknown sequence (Appendix A) had been recovered previously.

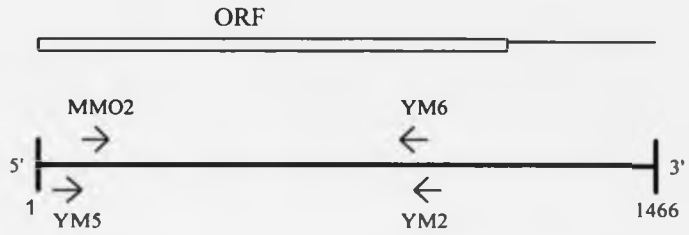
The two sets of primers used are detailed in Table 23 and Figure 43. The EdM sequence (III.2.2) was used to design the primers used for the amplifications. The first set of primers (YM5 and YM2) was designed to amplify a 1000 base fragment of the M gene (PCR A). The second set of primers (MMO2 and YM6) was designed so that they anneal at 5' and 3' regions of the 1000 base fragment previously amplified, thus the second PCR reaction should amplify a fragment of approximately 900 bases (PCR B) (Figures 44 & 45). Amplification of the M gene had been previously carried out using Vent polymerase, and the nested PCR reactions were initially tried using this enzyme. However, the controls for PCR B (see below) repeatedly produced a fragment of the expected size, therefore, amplification reactions using Taq polymerase were tried. The controls for PCR B remained clear, thus, the amplified products using Taq polymerase were cloned.

Table 23. Nested PCR primers for the YM-V M gene

Name	Position	Sequence	Restriction Sites	Tm °C
YM5	5 - 22	5' GCA AAG TGA TTG CCT CCC 3'	Pst I	56
YM2	1000 - 982	3' CCG CAT TTA CGA CGA CGT G 5'	Sal I	60
MMO2	22 - 44	5' GTT CCA CAA TGA CAG AGA TC 3'	Xho I	58
YM6	925 - 908	3' GCT CCT CCA GAG TAA TCG 5'	Ava I	56

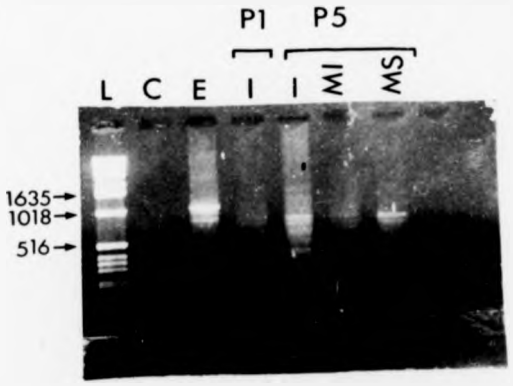
The primers were designed using the published M gene for the Edmonston strain of measles virus (Bellini *et al.*, 1986). Restriction sites were included in the primers to aid any subsequent cloning reactions. The cycle used for each PCR reaction was 94°Cx45 secs, 56°Cx45 secs and 74°Cx1 min (30 cycles).

Figure 43. Position of the primers within the M gene for the nested PCR



Nested PCR was required to increase the sensitivity of the reaction. YM5/YM2 primer pairs were positioned to initially amplify a 1 kb fragment. The resulting PCR reaction was then amplified using the MMO2/YM6 primer pair. This primer pair was positioned just inside the 5' and 3' ends of the 1 kb fragment, resulting in the amplification of a 900 base fragment.

Figure 44. M gene fragments amplified by PCR with primers YM5 and YM2



L: 1 kb ladder; C: negative control; E: ADD-MV, positive control; I P1: virus passaged once in IMR-32 cells; I P5: virus passaged 5 times in IMR-32 cells; MI P5: virus grown in MRC-5 cells followed by 5 passages in IMR-32 cells: MS P5: virus grown in MRC-5 cells, followed by 5 passages in SK-N-SH cells. PCR reaction A using the YM5/YM2 primer pair (II.3.3.3). Cycle used: 94°Cx45 secs, 56°Cx45 secs and 74°Cx1 min (30 cycles). A 3 µl aliquot of the completed PCR product was used in PCR B.

PCR A was tried using the MMO1/YMP primer pair. This pairing would rely on the amplification of bicistronic cDNA as YMP primes in the UTR of the P gene. This reaction did not work well presumably due to base changes at 3' region of the YM-V M gene which prevented the MMO1 primer annealing or low abundance of bicistronic cDNA. Therefore, the YM5/YM2 primer pair was used for subsequent reactions.

A negative control i.e. no cDNA was run for each PCR reaction. For PCR B a further control was run using the PCR product of the negative control for PCR A. This ensured

Figure 45. M gene fragments amplified by PCR with primers MMO2 and YM6

L: 1 kb ladder; C1: negative control for this reaction; C2: negative control from PCR A; E: ADD-MV, positive control; I P1: virus passaged once in IMR-32 cells; I P5: virus passaged 5 times in IMR-32 cells; MI P5: virus grown in MRC-5 cells followed by 5 passages in IMR-32 cells: MS P5: virus grown in MRC-5 cells, followed by 5 passages in SK-N-SH cells. PCR B used the MMO2/YM6 primer pair and cycle used was 94°Cx45 secs, 56°Cx45 secs and 74°Cx1 min (30 cycles) (II.3.3.3). Half of the PCR product (50 µl) was electrophoresed on a 1% agarose TAE gel. The 900 base fragment was excised from the and underwent Geneclean II procedure prior to cloning into the TA vector.

that there was no low level contamination of the PCR reagents used in the experiment. ADD-MV propagated in Vero cells was used as a positive control. From Figure 44, showing the results of PCR A, it can be seen that the positive control amplifies a fragment of 1000 bases as expected. The 4 samples of YM-V produce a number of smaller fragments, the largest and by far the clearest fragment being approximately 900 bases. The PCR products obtained from PCR B are much cleaner (Figure 45). No fragments were amplified from the negative controls and all 4 YM-V samples and the positive control have show fragments of approximately 900 bases as expected. The M gene of YM-V passaged once and 5 times in IMR-32 cells; YM-V passaged in MRC-5 cells followed by 5 passages in IMR-32 cells, and YM-V passaged in MRC-5 cells followed by 5 passages in SK-N-SH cells were then cloned into the TA vector (Promega).

III.3.7.2 Cloning the M Gene of YM-V into the TA Vector

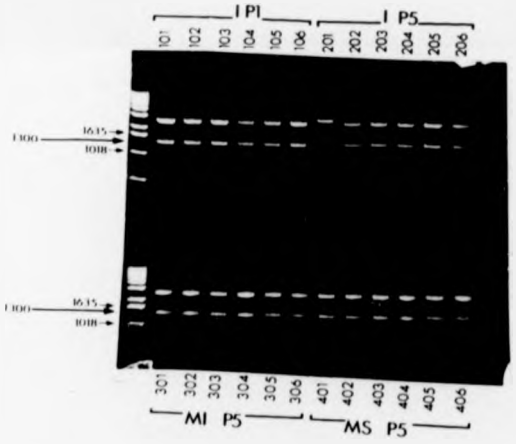
The TA vector, pGEMT (Promega), was used for this experiment. PCR using Taq DNA polymerase generates an overhanging A nucleotide at the end of the PCR product. TA vectors have a complementary T base which base pairs with the overhanging A on the PCR product, as such, there is no need to digest the PCR product or the vector prior to cloning. The PCR products were subjected to gel purification procedures and the cleaned products were ligated into the vector. Positive clones were grown up overnight and the double-stranded DNA was prepared using the silica particle method (II.7.2). The double-stranded DNA was digested with Pvu II to determine which clones contained the insert. Figure 46 shows the positive pGEMT clones of the M gene of YM-V passaged once and

five times in IMR-32 cells; propagated in MRC-5 cells prior to five passages in IMR-32 cells, and propagated in MRC-5 cells prior to five passages in SK-N-SH cells.

III.3.7.3 Sequencing of the M gene pGEMT Clones

The internal primers used for sequencing the double-stranded DNA have already been described (III.2.4.1) The primers used were -40, MM04 and MM07.

Figure 46. Positive pGEMT clones of the M gene of YM-V



L: 1kb ladder; I P1: virus passaged once in IMR-32 cells; I P5: virus passaged 5 times in IMR-32 cells; MI P5: virus grown in MRC-5 cells, followed by 5 passages in IMR-32 cells; MS P5: virus grown in MRC-5 cells, followed by 5 passages in SK-N-SH cells. 101 etc.: clone ID number. The PCR products were cloned into the pGEMT vector by virtue of the A overhang produced from the Taq polymerase amplification. DNA from clones were prepared by the silica particle method (II.7.2) and digested with Pvu II, therefore, actual size of clone is approx. 200 bp smaller than that represented on figure 46.

Three clones of each of the passaged virus M gene were sequenced once in full using the double-stranded sequencing method (II.8.3), and once in full using the automated sequencer (II.8.1.1). Double-stranded DNA was sequenced, therefore, primers that anneal to the other strand could have been used. However, as the two methods of sequencing i.e., automated sequencing and manual double-stranded sequencing were employed, confirmation of the sequence did not need to be done using the second strand, the alternative sequencing method sufficed.

III.3.7.4 M Gene Sequence of YM-V Passaged Once in IMR-32 Cells

The M gene of YM-V passaged once in IMR-32 cells was amplified by nested PCR, cloned into the pGEMT vector and three of the resulting clones were sequenced (900 bases). Each of the three M gene clones (nos. 101, 102 & 103) contained sequence resembling the M gene of Yamagata-1 virus described by Wong *et al.* (1989), except for the U-C base transition at positions 26, 34, 173, 180 and 182. The U-C transition described by Wong *et al.* (1989) at position 34 mutates the translational start codon. However, these three clones have a normal initiation codon, thus, a full length M protein could be translated, although the amino acid sequence will be different from that of acute measles virus M gene. The mutation which destroys the translation initiation codon, however, is present in the M gene fragments that were cloned into M13mp18 (III.3.5.4), whereas the U-C transitions observed by Wong *et al.* (1989) at positions 173, 180 and 182 do not occur in YM-V M gene fragments cloned into either M13mp18 (III.3.5.4) or pGEMT.

Clone 101 contains 3 additional base changes not described by Wong *et al.* (1989). These are at positions 494, a G-A transition, 751, an A-G transition, and 802, an A-G change (Table 24).

Clone 102 also contains the G-A base transition at position 494, and the U-C transition at position 108 that was present in clones 101 and 102, was not seen in clone 103 (Table 24).

Table 24. Sequence changes observed when comparing clones 101, 102 and 103 of YM-V passaged once in IMR-32 cells with the M gene of Yamagata-1 virus described by Wong *et al.* (1989)

Clone 101		Clone 102		Clone 103	
Position	Base Change	Position	Base Change	Position	Base Change
26	C-U	26	C-U	26	C-U
34	C-U	34	C-U	34	C-U
45	C-U	-	-	45	C-U
-	-	-	-	108	C-U
173	C-U	173	C-U	173	C-U
180	C-U	180	C-U	180	C-U
182	C-U	182	C-U	182	C-U
494	G-A	494	G-A	-	-
751	A-G	-	-	-	-
802	A-G	-	-	-	-

The M gene of YM-V passaged once in IMR-32 cells was amplified by nested PCR and cloned into the pGEMT vector. Three clones were sequenced using manual and automated sequencing methods. The sequence of the 3 clones was compared with the M gene of Yamagata-1 virus propagated in Vero cells only (Wong *et al.*, 1989) and the base changes observed are tabulated above.

III.3.7.5 M Gene Sequence of YM-V passaged 5 Times in IMR-32 Cells

The M gene of YM-V passaged 5 times in IMR-32 cells was amplified by nested PCR, cloned and sequenced. Three clones (nos. 202, 203 & 204) were sequenced in full (900 bases). Clone 204 contains most of the base changes described by Wong *et al.* (1989)

(Table 25), except that three base changes are present that were not previously, namely a C-A nucleotide substitution at position 755, a G-A transition at position 493, and an A-U transversion at position 354. The uracil to cytosine transition at positions 173, 180 and 181 are not present in these three clones either, nor is the U-C transition at position 34, which destroys the translation start codon. The intact translation initiation codon suggests that a full length M protein could be translated from these clones. These three clones are also missing the U-C base transition at position 26 which Wong *et al.* (1989) observed.

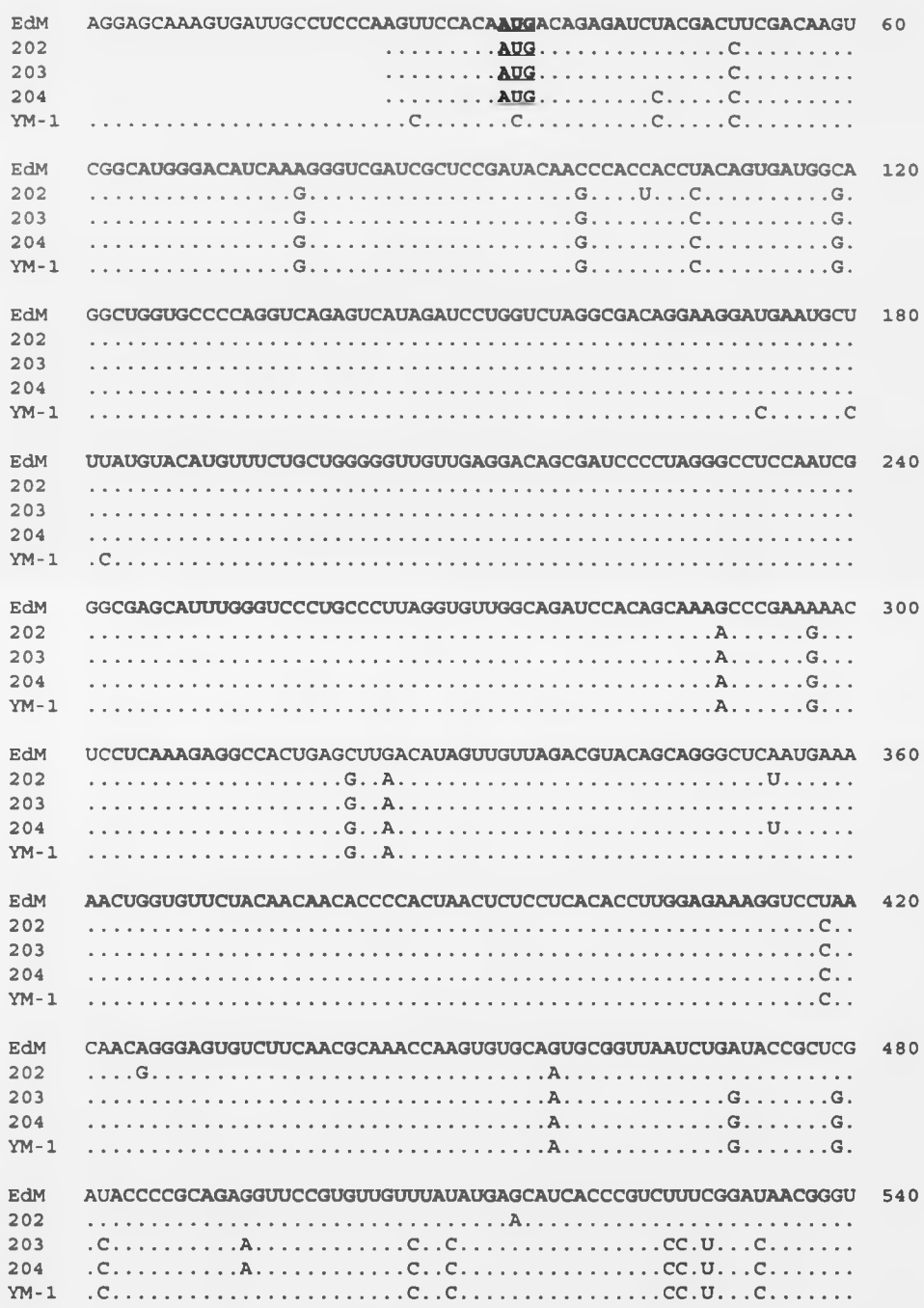
Clones 202 and 203 are exceptional in that they appear to be chimeras of Yamagata-1 virus M gene and EdM. Clone 202 contains the M gene sequence of Yamagata-1 virus up to position 457, after this position the sequence reverts to that of EdM (III.2.2). This clone also contains 4 additional base changes at positions 104 (C-U), 354 (A-U), 425 (A-G) and at position 514 (G-A). Clone 203 contains the base changes observed in the Yamagata-1 virus up to position 746, then the M gene sequence resembles EdM except for the U-C transition at position 883, which was described by Wong *et al.* (1989) in the Yamagata-1 virus. This clone contains one additional base changes at position 493 (G-A). Figure 47 shows the sequence obtained from the three clones.

Table 25. Comparison of the sequences of the three clones from YM-V passaged 5 times in IMR-32 with the M gene of Yamagata-1 virus described by Wong *et al.* (1989)

Clone 202		Clone 203		Clone 204	
Position	Base Change	Position	Base Change	Position	Base Change
26	C-U	26	C-U	26	C-U
34	C-U	34	C-U	34	C-U
45	C-U	45	C-U	-	-
104	C-U	-	-	-	-
173	C-U	173	C-U	173	C-U
180	C-U	180	C-U	180	C-U
182	C-U	182	C-U	182	C-U
354	A-U	493	G-A	354	A-U
425	A-G	-	-	493	G-A
*457	*G-A	-	-	755	C-A
479	G-C	-	-	-	-
482	C-U	-	-	-	-
506	C-U	-	-	-	-
509	C-U	-	-	-	-
514	G-A	-	-	-	-
526	C-U	-	-	-	-
527	C-U	-	-	-	-
529	U-C	-	-	-	-
533	C-U	-	-	-	-
542	C-U	-	-	-	-
554	C-U	-	-	-	-
575	G-A	-	-	-	-
617	A-G	-	-	-	-
632	U-A	-	-	-	-
657	G-A	-	-	-	-
699	G-A	-	-	-	-
746	G-A	*	*	-	-
811	C-U	811	C-U	-	-
871	C-U	871	C-U	-	-
881	A-G	881	A-G	-	-
883	C-U	-	-	-	-
884	A-G	884	A-G	-	-
904	C-U	904	C-U	-	-
905	C-U	905	C-U	-	-

The M gene of YM-V passaged 5 times in IMR-32 cells was amplified by nested PCR and cloned into the pGEMT vector. Three clones were sequenced using manual and automated sequencing methods. The sequence of the 3 clones was compared with the M gene of Yamagata-1 virus propagated in Vero cells only (Wong *et al.*, 1989) and the base changes observed are tabulated above. * Denotes position in clone where the sequence reverts to that of EdM.

Figure 47. M gene sequence of YM-V passaged 5 times in IMR-32 cells



```
EdM   AUUACACCGUUCCUAGAAGAAUGCUGGAAUUCAGAUCGGUCAAUGCAGUGGCCUUCAACC  600
202   ....................................................................
203   .C..........C.....................G.....................................
204   .C..........C.....................G.....................................
YM-1  .C..........C.....................G.....................................

EdM   UGCUGGUGACCCUUAGGAUUGACAAGGCGAUAGGCCCUGGGAAGAUCAUCGACAAUACAG  660
202   ....................................................................
203   ................A.............U.........................G...
204   ................A.............U.........................G...
YM-1  ................A.............U.........................G...

EdM   AGCAACUUCCUGAGGCAACAUUUAUGGUCCACAUCGGGAACUUCAGGAGAAAGAAGAGUG  720
202   ....................................................................
203   ....................................G.....................
204   ....................................G.....................
YM-1  ....................................G.....................

EdM   AAGUCUACUCUGCCGAUUAUUGCAAAAUGAAAAUCGAAAAGAUGGGCCUGGUUUUUGCAC  780
202   ....................................................................
203   ......................G.............................................
204   ......................G.........A...................................
YM-1  ......................G.....................................

EdM   UUGGUGGGAUAGGGGGCACCAGUCUUCACAUUAGAAGCACAGGCAAAAUGAGCAAGACUC  840
202   ....................................................................
203   ....................................................................
204   ............................C.......................................
YM-1  ............................C.......................................

EdM   UCCAUGCACAACUCGGGUUCAAGAAGACCUUAUGUUACCCGCUGAUGGAUAUCAAUGAAG  900
202   ....................................................................
203   ..........................................C.........................
204   ............................C.........A.CA.........................
YM-1  ............................C.........A.CA.........................

EdM   ACCUUAAUCGAUUACUCUGGAGGAGC                                      926
202   ........................
203   ........................
204   ...CC...................
YM-1  ...CC...................
```

EdM correspond to the published M gene sequence (Bellini *et al.*, 1986). 202, 203 and 204 are the 3 clones sequences of YM-V passaged 5 times in IMR-32 cells, and YM-1 is the M gene of Yamagata-1 virus passaged in Vero cells only observed by Wong *et al.* (1989). Clones 202 and 203 are chimeras containing both EdM and YM-1 sequence. The sequence was determined using both manual and automated sequencing methods. **AUG** is the translation initiation codon. The open reading frame extends past the 960 bases sequence, therefore, it is presumed that a full length M protein is translated.

III.3.7.6 M Gene Sequence of YM-V Propagated in MRC-5 Cells Followed by 5 Passages in IMR-32 Cells

The M gene of YM-V passaged 3 times in MRC-5 cells followed by 5 passaged in IMR-32 cells was amplified by nested PCR, cloned into pGEMT and sequenced. Three clones (301, 302 & 302) of this passaged virus were sequenced in full (900 bases). The U-C base transition at position 34, which destroys the translation initiation codon, in the M gene Yamagata-1 virus (Wong *et al.*, 1989) was not observed in any of these clones. Clone 301 resembles the M gene Yamagata-1 virus (Wong *et al.*, 1989), except that the five U-C transitions at positions 26, 45, 173, 180 and 182 were not present. Two additional mutations were observed; one was at position 345 (G-A), and the other was at position 797 (C-A). Clone 302 appears to be a chimera having sequence resembling EdM up to position 457 (G-A), beyond which the sequence reverts to that described by Wong *et al.* (1989). This clone contains 3 additional mutations, one at position 220 (A-G), one at position 741 (U-C), and one at position 831 (A-G). Clone 303 contains sequence which resembles EdM. Figure 48 shows the sequence of these 3 clones; Table 26 summarises the base differences when clones 301 and 302 are compared to the M gene of Yamagata-1 virus determined by Wong *et al.* (1989).

Figure 48. M gene sequence of YM-V passaged in MRC-5 cells followed by 5 passages in IMR-32 cells

```
EdM    AGGAGCAAAGUGAUUGCCUCCCAAGUUCCACAAUGACAGAGAUCUACGACUUCGACAAGU  60
301                              .........AUG.............C.........
302                              .........AUG.............C.........
303                              .........AUG.............C.........
YM-1   ........................C.......C..........C.....C.........

EdM    CGGCAUGGGACAUCAAAGGGUCGAUCGCUCCGAUACAACCCACCACCUACAGUGAUGGCA  120
301    ................G.....................G........C.........G.
302    ...............................................................
303    ...............................................................
YM-1   ................G.....................G........C.........G.

EdM    GGCUGGUGCCCCAGGUCAGAGUCAUAGAUCCUGGUCUAGGCGACAGGAAGGAUGAAUGCU  180
301    ...............................................................
302    ...............................................................
303    ...............................................................
YM-1   ...............................................................C.....C

EdM    UUAUGUACAUGUUUCUGCUGGGGGUUGUUGAGGACAGCGAUCCCCUAGGGCCUCCAAUCG  240
301    ...............................................................
302    ..........................................G.U..................
303    ..........................................U....................
YM-1   .C.............................................................

EdM    GGCGAGCAUUUGGGUCCCUGCCCUUAGGUGUUGGCAGAUCCACAGCAAAGCCCGAAAAAC  300
301    ...............................................A......G...
302    ...............................................................
303    ...............................................................
YM-1   ...............................................A......G...

EdM    UCCUCAAAGAGGCCACUGAGCUUGACAUAGUUGUUAGACGUACAGCAGGGCUCAAUGAAA  360
301    ..................G..A.....................A.................
302    ...............................................................
303    ...............................................................
YM-1   ..................G..A.....................................

EdM    AACUGGUGUUCUACAACAACACCCCACUAACUCUCCCUCACACCUUGGAGAAAGGUCCUAA  420
301    ...............................................................C..
302    ...............................................................C..
303    ...............................................................
YM-1   ...............................................................C..

EdM    CAACAGGGAGUGUCUUCAACGCAAACCAAGUGUGCAGUGCGGUUAAUCUGAUACCGCUCG  480
301    ...................................A.............G.......G.
302    ...................................A.............G.......G.
303    ...................................A.....................
YM-1   ...................................A.............G.......G.
```

```
EdM   AUACCCCGCAGAGGUUCCGUGUUGUUUAUAUGAGCAUCACCCGUCUUUCGGAUAACGGGU  540
301   .C.....................C..C.............CC.U...C.......
302   .C.....................C..C.............CC.U...C.......
303   ........................................................
YM-1  .C.....................C..C.............CC.U...C.......

EdM   AUUACACCGUUCCUAGAAGAAUGCUGGAAUUCAGAUCGGUCAAUGCAGUGGCCUUCAACC  600
301   .C..........C.....................G.....................
302   .C..........C.....................G.....................
303   ........................................................
YM-1  .C..........C.....................G.....................

EdM   UGCUGGUGACCCUUAGGAUUGACAAGGCGAUAGGCCCUGGGAAGAUCAUCGACAAUACAG  660
301   ..............A.............U.......................G...
302   ..............A.............U.......................G...
303   ........................................................
YM-1  ..............A.............U.......................G...

EdM   AGCAACUUCCUGAGGCAACAUUUAUGGUCCACAUCGGGAACUUCAGGAGAAAGAAGAGUG  720
301   ....................................G...................
302   ....................................G...................
303   ........................................................
YM-1  ....................................G...................

EdM   AAGUCUACUCUGCCGAUUAUUGCAAAAUGAAAAUCGAAAAGAUGGGCCUGGUUUUUGCAC  780
301   ......................G.................................
302   ..................C....G.................................
303   ........................................................
YM-1  ......................G.................................

EdM   UUGGUGGGAUAGGGGGCACCAGUCUUCACAUUAGAAGCACAGGCAAAAUGAGCAAGACUC  840
301   ..............A.............C...........................
302   ..............................C.................G.......
303   ........................................................
YM-1  ..............................C...........................

EdM   UCCAUGCACAACUCGGGUUCAAGAAGACCUUAUGUUACCCGCUGAUGGAUAUCAAUGAAG  900
301   ..............................C.........A.CA.............
302   ..............................C.........A.CA.............
303   ........................................................
YM-1  ..............................C.........A.CA.............

EdM   ACCUUAAUCGAUUACUCUGGAGGAGC                                  926
301   ...CC..................
302   ...CC..................
303   ......................
YM-1  ...CC..................
```

EdM correspond to the published M gene sequence (Bellini *et al.*, 1986). 301, 302 and 303 are the 3 clones sequences of the M gene of YM-V passaged in MRC-5 cells followed by 5 passaged in IMR-32, and YM-1 is the M gene of Yamagata-1 virus passaged in Vero cells only observed by Wong *et al.*, 1989. The sequence was determined using both automated and manual sequencing methods. Clone 302 appears to be a chimera containing both EdM and YM-1 sequence. **AUG** is the translation initiation codon. The open reading frame extends past the 960 bases sequence, therefore, it is presumed that a full length M protein is translated.

Table 26. Comparison of sequence of clones 301 and 302 of the M gene of YM-V propagated in MRC-5 cell followed by 5 passages in IMR-32 cells with the M gene of Yamagata-1 virus described by Wong *et al.* (1989)

Clone 301		Clone 302	
Position	Base Change	Position	Base Change
26	C-U	26	C-U
34	C-U	34	C-U
45	C-U	45	C-U
-	-	51	C-U
-	-	77	A-G
-	-	99	G-C
-	-	108	C-U
-	-	119	G-C
173	C-U	173	C-U
180	C-U	180	C-U
182	C-U	182	C-U
-	-	220	A-G
-	-	222	C-U
-	-	290	A-G
-	-	297	G-A
-	-	321	G-C
345	G-A	324	A-G
-	-	418	C-U
-	-	*457	-
-	-	741	U-C
797	C-A	831	A-G

The M gene of YM-V propagated in MRC-5 cells, followed by 5 passage in IMR-32 cells was amplified by nested PCR and cloned into the pGEMT vector. Three clones were sequenced using manual and automated sequencing methods. The sequence of the 3 clones was compared with the M gene of Yamagata-1 virus propagated in Vero cells only (Wong *et al.*, 1989) and the base changes observed are tabulated above. * indicates the position at which the sequence for clone 302 reverts from EdM to the Yamagata-1 sequence determined by Wong *et al* (1989).

III.3.7.7 M Gene Sequence of YM-V in MRC-5 Cells Followed by 5 Passages in SK-N-SH Cells

The M gene of YM-V passaged 3 times in MRC-5 cells followed by 5 passages in SK-N-SH cells was amplified by nested PCR, cloned into pGEMT and sequenced. Three clones (401, 402 & 403) of this passaged virus were sequenced in full (900 bases). Clone 401

contained sequence resembling EdM. Clones 402 and 403 contained sequence resembling the M gene of Yamagata-1 (Wong *et al.*, 1989), except that the translation initiation codon of these clones is not mutated (position 34). These two clones (402 and 403) are also lacking 4 U-C base transitions at positions 26, 173, 180 and 182, which were described by Wong *et al.* (1989). A transversion at position 354 (A-U) is also present in these clones. Clone 402 contained one other base change at position 45. The U-C transition observed by Wong *et al.* (1989) at this position is not present in this clone. Clone 403 contains one other transition when compared to the M gene of Yamagata-1 virus, at position 73 (U-C). Tables 27 and 28 summarises the differences in the sequences of these three clones when compared to either EdM (III.2.2) or the M gene of Yamagata-1 virus determined by Wong *et al.* (1989).

Table 27. Comparison of the M gene sequence of clones 402 and 403 of YM-V propagated in MRC-5 cells followed by 5 passages in SK-N-SH cells with the M gene of Yamagata-1 virus described by Wong *et al.* (1989)

Clone 402		Clone 403	
Position	Base Change	Position	Base Change
26	C-U	26	C-U
34	C-U	34	C-U
45	C-U	-	-
-	-	73	U-C
173	C-U	173	C-U
180	C-U	180	C-U
182	C-U	182	C-U
354	A-U	354	A-U

The M gene of YM-V propagated in MRC-5 cells followed by 5 passages in SK-N-SH cells was amplified by nested PCR and cloned into the pGEMT vector. Three clones were sequenced using manual and automated sequencing methods. The sequence of clones 402 and 403 was compared with the M gene of Yamagata-1 virus propagated in Vero cells only (Wong *et al.*, 1989) and the base changes observed are tabulated above.

Table 28. Comparison of the sequence of the M gene clone (401) of YM-V propagated in MRC-5 cells followed by 5 passages in SK-N-SH cells with EdM

Clone 401	
Position	Base Change
48	G-U
61	C-U
222	C-U
457	G-A

The M gene of YM-V propagated in MRC-5 cells, followed by 5 passaged in SK-N-SH cells was amplified by nested PCR and cloned into the pGEMT vector. Three clones were sequenced using manual and automated sequencing methods. The sequence clone 401 was compared with the published M gene sequence of the Edmonston strain of measles virus (Bellini *et al.*, 1986) and the base changes observed are tabulated above.

III.3.8 Conclusions and Discussion

III.3.8.1 Summary of Results

The following tables summarise the results of the M gene cloning reactions from the YM-V passage experiment. Table 29 presents the M13 cloning results, and Table 30 those M gene fragments cloned into pGEMT.

Cloning of the M gene of YM-V has revealed that the initial virus stock provided by Professor Homma contains a heterogeneous virus population consisting of both acute measles virus M gene (EdM) and Yamagata-1 virus M gene sequences. One of the aims of this project was to determine whether passage in neuroblastoma cells alone was enough to induce hypermutational events. In the present work, comparing the M gene sequence determined from YM-V passaged once in the neuroblastoma cell lines with that from YM-V passaged five times did not reveal any additional hypermutation events. In the study carried out by Wong *et al.* (1989) the sequence of the M gene of Yamagata-1

Table 29. Summary of the YM-V M gene clones which were amplified by PCR and cloned into the bacteriophage M13

	Passage 1			Passage 3	Passage 5			
Cell type	MRC-5 SK-N-SH	IMR-32	MRC-5 IMR-32	SK-N-SH	SK-N-SH	MRC-5 SK-N-SH	IMR-32	MRC-5 IMR-32
No. Clones	10	25	4	11	19	29	18	21
Measles Virus	5	-	4	11	4	-	-	-
Unknown	5*	25	-	-	15	29	18	19
Homo Sapiens cDNA clone	-	-	-	-	-	-	-	1
H influenzae recF,dnaF etc.	-	-	-	-	-	-	-	1

The M gene of Yamagata-1 virus, passaged as indicated above, was amplified using the YMP/YM2 primer pair. The resulting 1 kb fragment was cloned into the bacteriophage M13 using Sal I and Hind III restriction site. Clones were sequenced initially using the -40 primer provided with the Sequenase v2.0 sequencing kit (USB) to determine whether they resembled measles virus M gene sequence, the unknown sequence (Appendix A) or artefacts produced from the cloning reaction. *1/5 unknown sequence (Appendix A); 4/5 artefacts produced during amplification and cloning reactions.

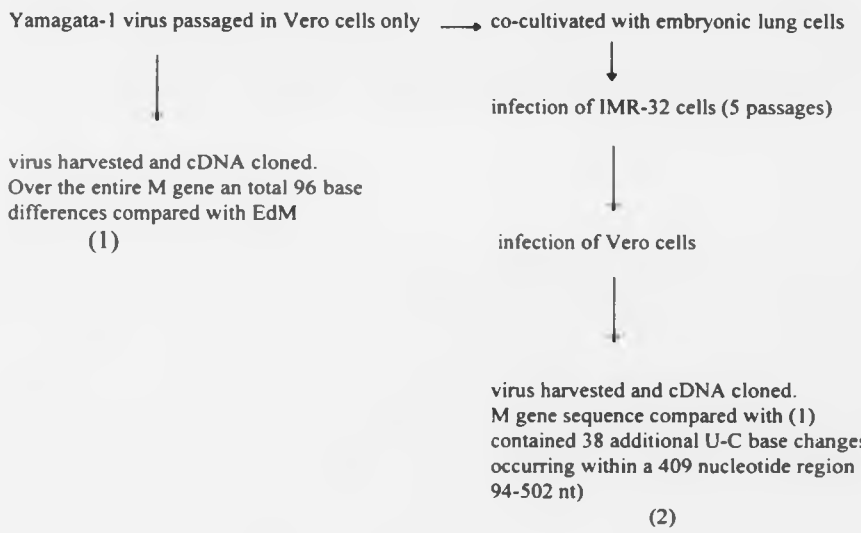
virus propagated in Vero cells differed by 41 bases within the first 926 bases of the M gene when compared with EdM. The M gene cloned from Yamagata-1 virus passaged 5 times in IMR-32 cells contained an additional 38 U-C transitions between bases 90 and 510. A biased hypermutational event was hypothesised to have occurred as a consequence of passage of the virus in the neuroblastoma cell line. The sequence data obtained from the passage of YM-V in the experiments described here did not contain these additional mutations. Thus, it was not possible to repeat the observation reported by Wong *et al.* (1989) (Figure 49). The only type of sequence which was novel to YM-V passaged 5 times was the chimeric M gene sequence observed in YM-V passaged 5 times in IMR-32 cells and in YM-V propagated in MRC-5 cells, followed by 5 passages in IMR-32 cells.

Table 30. Summary of the YM-V M gene sequences which were amplified by both nested and traditional PCR methods

Virus Passage	Clone ID	EdM-like	YM-V-like	Chimera	Sequence length
YM-V	4		✓		1001 bases
	5	✓			1001 bases
	7	✓			1001 bases
S P3	8	✓			743 bases
	10	✓			743 bases
	18	✓			743 bases
S P5	2	✓			726 bases
	5	✓			726 bases
	18	✓			960 bases
MS P1	1	✓			960 bases
	11		✓		960 bases
	14		✓		960 bases
*MS P5	401	✓			900 bases
	402		✓		900 bases
	403		✓		900 bases
*I P1	101		✓		900 bases
	102		✓		900 bases
	103		✓		900 bases
*I P5	202			✓	900 bases
	203			✓	900 bases
	204		✓		900 bases
MI P1	1		✓		960 bases
	2		✓		960 bases
	6		✓		960 bases
*MI P5	301		✓		900 bases
	302			✓	900 bases
	303	✓			900 bases

YM-V: virus passaged 102 times in Vero cells; I P1: virus passaged once in IMR-32 cells; I P5: virus passaged 5 times in IMR-32 cells; MI P1: virus grown in MRC-5 cells followed by once passage in IMR-32 cells; MI P5: virus grown in MRC-5 cells, followed by 5 passages in IMR-32 cells; S P3: virus passaged 3 times in SK-N-SH cells; S P5: virus passaged 5 times in SK-N-SH cells; MS P1: virus grown in MRC-5 cells, followed by one passaged in SK-N-SH cells; MS P5: virus grown in MRC-5 cells, followed by 5 passages in SK-N-SH cells. M gene of YM-V was amplified using the YMP/YM2 primer pair and cloned into the bacteriophage M13. The sequence was determined using the Sequenase sequencing kit (USB). * indicates M gene products that were amplified using nested PCR (YM5/YM2 and MMO2/YM6 primer pairs). The resulting fragment was cloned into the pGEMT vector (Promega) and sequenced using both manual and automated methods.

Figure 49. Summary of the M gene sequence determined by Wong *et al.* (1989)



The Yamagata-1 virus passaged protocol and a summary of the results obtained by Wong *et al.* (1989). poly A^+ mRNA was reverse transcribed to cDNA using oligo T primer. The resulting cDNA was cloned and sequenced. Four further clones of IMR-32 passaged virus were evaluated for restriction enzyme polymorphism which showed the presence of at least two distinct M gene populations, although no further sequencing was carried out on these clones.

Furthermore, propagation of YM-V in human diploid cells prior to passage in the neuroblastoma cell lines did not have any effect on the propensity for hypermutational events. There was no difference between the sequences obtained from YM-V which had been grown in MRC-5 cells before passaged in IMR-32 cells or SK-N-SH cells when compared with YM-V which had only been passaged in the neuroblastoma cells.

Two clones from YM-V passaged 5 times in IMR-32 cells (I P5 clones 202 and 203) and one clone from YM-V propagated in MRC-5 prior to 5 passages in IMR-32 cells (MI P5 clone 302) were chimeras containing both EdM-like sequence and the M gene sequence of Yamagata-1 virus (Wong *et al.*, 1989). This suggests that recombination between

acute measles virus M gene and the mutated M gene associated with SSPE viruses had occurred.

III.3.8.2 Sequence Anomalies

The sequence data derived from three clones from YM-V passaged 3 times in SK-N-SH was 743 bases, whereas both the amplified product (Figure 37) and the inserted fragment in the vector M13 (Figure 38) was shown to be 1000 bases as expected. The reason for this discrepancy is unclear. A possible explanation is that the oligo dT primer used to synthesize the cDNA primed at the position 743/ AAAATGAAAATCGAAAA /760. The mRNA:cDNA hybrid formed after cDNA synthesis would be 760 bases long. The YMP sequence would anneal to the negative sense cDNA, and amplify a fragment of approximately 760 bases which would contain Hind III ER site at both the 3' and 5' end because the oligo dT primer contained the Hind III ER site which was one of the enzymes used to clone the PCR fragments. One problem with this interpretation is that both the amplified product and the insert were shown to be 1000 bases (Figures 38 & 40) not 760 bp which would be expected. If oligo dT mis-priming had occurred, the vector stock may not be contaminated with M13mp19. The PCR fragment would contain Hind III sites at both ends, therefore, the exact orientation of the insert could not be ascertained until the positive clone was sequenced. However, the vector was digested with both Hind III and Sal I so an insert containing Hind III ER sites at both ends would not ligate into this vector.

Sequencing errors occurring at position 743/ AAAATGAAAATCGAAAA /760 might explain this anomaly. Errors during the sequencing reaction could reduce this sequence to a poly A tail, however, the 11 bases preceding the poly A tail (Figure 42) do not correspond with either the YM2 primer nor the M gene sequence directly after base 760.

The YM2 primer sequence was not observed in any of the 3 clones for this virus passage. It was possible, therefore, that contamination of the M13 vector stock with the ADD-MV has occurred. The observation that the sequence of these 3 clones is exactly the same length as that determined for the M gene of ADD-MV passaged once in each of the four cell lines favours this, but does not account for the size of the insert being 1000 bases as expected (Figure 38).

It is also possible that the -40 primer has mis-primed. At position 957 to 941 of the M gene there is 53% homology with the -40 primer. This is not a high identity overall, however, the 3'-end of the primer, which requires strong homology in order for the entire primer to anneal, has 100% identity over 5 bases. As such the -40 could have annealed at this position. This still does not explain why bases 744 to 941 were not determine, however, this could have been resolved by electrophoresis of the -40 sequencing reaction for <2 hrs in order to try to determine sequence nearer the primer. Also the deaza-reactants in the sequencing kit could have been used in order to sequence nearer the primer.

Two clones from YM-V passaged 5 times in SK-N-SH produced sequence that was 726 bases instead of the expected 1000 bases. The most likely explanation for this is the

introduction of a Sal I restriction site due to a U-G base change at position 726. As this ER site was used in the cloning procedure the amplified fragment to be cloned would be digested at this position, so the inserted sequence would be of a reduced size.

III.3.8.3 Discussion

The observation that the original virus stock was a heterogeneous population was made after YM-V passage in the neuroblastoma cells had been initiated and the cloning of passage one had been completed. Had the passage and cloning of YM-V not been so advanced, a more controlled experiment would have been carried out in which the YM-V stock was plaque-purified and then passaged. The M gene sequence of the biologically cloned virus would have to have been determined to ensure that the material for passage was the same genotype as that described by Wong *et al.* (1989).

It is possible that the passage protocol may have selected for virus with a lytic phenotype, but no assays were done to confirm this, such as titres of the virus after each passage. If a lytic phenotype was being positively selected one would expect to see an increase in virus titre during the passage experiments. However, for each passage two 25 cm^2 flasks were infected, one of which was freeze/thawed and aliquoted into 1 ml portions as an RNA source, the remaining flask was treated with EDTA/Trypsin and the dispersed cells used as the inoculum for subsequent passage. Thus, reinfection was mediated by cell-associated rather than released virus and selection for lytic virus seems unlikely.

A low titre, less than 1000 pfu/ml, was determined from YM-V passage 96. Homma *et al.* (1982) also reported a titre of less that 1000 pfu/ml from the newly isolated Yamagata-1 virus, and in the study carried out by Wong *et al.* (1989) a titre of $2x10^2$ pfu/ml was determined from virus passaged in the IMR-32 cell. Thus, it is probable that the Yamagata-1 virus isolated from autopsy material contained acute measles virus and that the EdM M gene sequence isolated in the experiments described here was not a consequence of contamination during the passaged of YM-V.

An independent study carried out by Baczko *et al.* (1993) detected both wild-type-like measles virus M gene sequence and hypermutated sequence in brain tissue from an SSPE patient. This information and the finding of this project, that EdM sequence can be detected in SSPE virus isolates, suggests that the acute measles virus M gene does not represents a contaminating virus. It is likely that the acute measles virus M gene detected represent the virus which initially infected the CNS of the patient from which Yamagata-1 virus was isolated. Virus populations with defective M genes are most likely selected for once the acute virus has entered neural tissue. Non-defective measles virus genomes are probably present in the brain during the progression of the disease, but at such low levels that they are not easily identified when extracting RNA directly from brain tissue.

In the study carried out by Bazcko *et al.* (1993), cDNA synthesised from RNA extracted from different regions of the brain was cloned directly. Fifteen percent of the clones sequenced were 'wild-type-like', the remainder contained the U-C transitions associated with SSPE virus. Of the 27 clones sequenced in this project, 41 % contained EdM-like M gene sequence; 48% contained the mutated M gene sequence associated with Yamagata-1

virus M gene (Wong *et al.*, 1989) and 11% were chimeras i.e., contained both EdM-like sequence and the mutated M gene sequence determined by Wong *et al.* (1989). The higher frequency of the EdM-like sequence may not be a true representation of the concentration of plaque-forming virus as the M gene sequence used for cloning was produced by PCR amplification. When the proportion of EdM clones and hypermutated M gene clones are compared using the regular PCR method (one set of primers) and nested PCR (two sets of primers), the more specific nested PCR method produced 58.3 % of clones containing the hypermutated gene; 16.6 % containing the EdM-like gene and 25% were chimeric clones. Whereas the cloning reaction where the M gene was amplified using the regular PCR method resulted in 40% of the clones containing hypermutated M gene sequence and 60% of the clones containing EdM-like sequence (Table 31). As such the nested PCR results correlate more closely with that of Baczko *et al.*, (1993).

Mutated sequences obtained from M genes cloned into both M13 and pGEMT did not contain the U-C base changes at nucleotide positions 173, 180 and 182 observed by Wong *et al.* (1989). The clones isolated in this experiment were prepared by amplifying the M gene of the passaged virus after cDNA synthesis, therefore, the most abundant M gene variant present in virus population was cloned. Although some small variation was seen within the clones isolated and sequenced they all resembled the M gene sequence of Yamagata-1 virus propagated in Vero cells (Wong *et al.*, 1989) or the EdM (III.2.2) sequence. The sequence of virus passaged in the IMR-32 cells, as determined by Wong *et al.* (1989) contained 38 additional base changes when compared to virus passaged only in Vero cells. However, the clones sequenced in that study were from cDNA, so a more

precise picture of the pre-existing variants would be observed. The M gene sequences determined in virus passaged 5 times in the neuroblastoma cell lines in the experiment described in this thesis were also detected in the YM-V starting material, except the chimeric species. It is likely, therefore, that the hypermutated clone sequenced after 5 passages in IMR-32 determined by Wong *et al.* (1989) represents a pre-existing variant which is not the predominant variant of M gene within the virus population, explaining why it was not detected during the experiments detailed in this thesis.

Recovery of the unknown sequence (Appendix A) from various virus passages was not fully examined. The attempts at identification of this material are discussed in more detail in appendix A. The use of nested PCR to amplify the M gene of YM-V was successful. The nested PCR M gene fragments cloned into the pGEMT vector were more heterogeneous than those cloned into M13. The translation initiation codons of these clones were not destroyed by a U-C mutation at position 34, unlike the M13 clones and the Yamagata-1 M gene sequence described by Wong *et al.* (1989). This suggests that intact M protein could be translated from the mutated M gene. The open reading frame (ORF) of such clones was maintained throughout the 950 base pair fragment sequenced. Baczko *et al.* (1993) also detected mutated M gene sequence both with and without the translation initiation codon. It is likely that a minimal population of YM-V-type virus is present which retains the ORF allowing the expression of the M gene. It is possible that the small number of base changes observed between the different clones probably represents polymerase errors during the amplification of the M gene, rather than pre-existing variants of the M gene within the virus population.

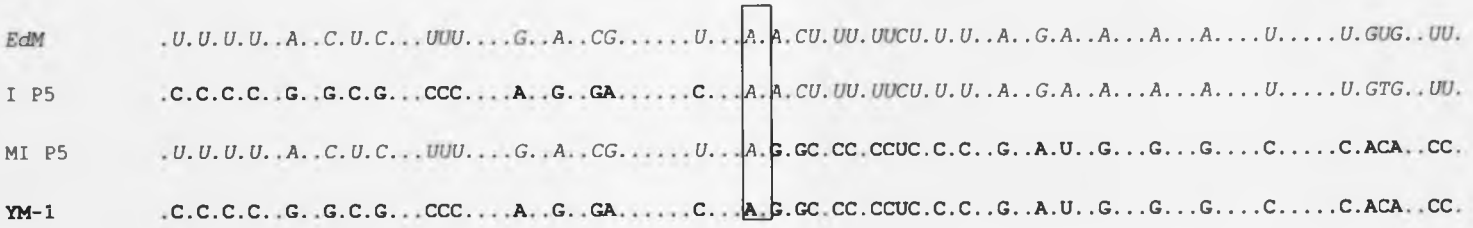
Table 31. Summary of the M gene sequences obtained from the YM-V passage in the neuroblastoma cell lines

		Passage 1			Passage 3	Passage 5			
Method of Amplification	Sequence Obtained	MRC-5 / SK-N-SH	IMR-32	MRC-5 / IMR-32	SK-N-SH	SK-N-SH	MRC-5 / SK-N-SH	IMR-32	MRC-5 / IMR-32
PCR	EdM-like	2	-	-	11	4	-	-	-
	YM-1-like	-	-	4	-	-	-	-	-
	Unknown	3	25	-	-	15	29	18	19
	Chimera	-	-	-	-	-	-	-	-
	Hypermutated YM-1	-	-	-	-	-	-	-	-
Nested PCR	EdM-like	-	-	-	-	-	1	-	1
	YM-1-like	-	3	-	-	-	2	1	1
	Unknown	-	-	-	-	-	-	-	-
	Chimera	-	-	-	-	-	-	2	1
	Hypermutated YM-1	-	-	-	-	-	-	-	-

This table summaries the M gene clones obtained from YM-V passaged in IMR-32 and SK-N-SH cells with and without prior passaged in MRC-5 cells. The M gene was amplified using the traditional PCR methodology (II.3.3.2) i.e., one set of PCR primer (YMP/YM2), and nested PCR (II.3.3.3) i.e., two sets of PCR primers (MMO2/YM6 & YM5/YM2). PCR products from the traditional PCR method were cloned into the bacteriophage M13 (II.6.3); nested PCR products were cloned into pGEMT (Promega) (II.6.4). M13 clones (single-stranded DNA)were sequenced using the Sequenase kit (II.8.2); pGEMT clones were sequenced using the double-stranded DNA sequencing method (II.8.3).

Two chimeric clones were detected; one contained M gene sequence that resembles the ADD-MV at the 5' end, and the Yamagata-1 virus M gene at the 3' region. Another clone contained ADD-MV M gene sequence at the 3' region, and Yamagata-1 M gene sequence at the 5' region (Figure 50). A common feature is that the base change at position 457 (G-A) was where the distinction between Yamagata-1 virus-like sequence and ADD-MV-like sequence occurred. This base change is one of the two observed in the M gene which differentiated the ADD-MV sequence from that described by Bellini *et al.* (1986). This may be the first evidence of intragenic recombination in measles virus.

Figure 50. Diagrammatic representation of the Edmonston-like region and the Yamagata-1-like regions of two clones



I P5: YM-V passaged 5 times in IMR-32 cells
MI P5: YM-V passaged 3 times in MRC-5 cells and 5 times in IMR-32 cells
YM-1: Region of clone that corresponds to the Yamagata-1 virus M gene sequence described by Wong *et al.* (1989)
Edm: Region of clone that corresponds to the EdM sequence.

The bases in *italics* correspond to the published M gene of the Edmonston strain of measles virus (Bellini *et al.*, 1986). The bases in **bold** correspond to the M gene sequence of Yamagata-1 virus passaged in Vero cells only, determined by Wong *et al.* (1989). The boxed bases indicate nucleotide position 457, where the sequence of the chimeric clones change from Edm: Yamagata-1 M gene or vice-versa.

PART IV
GENERAL DISCUSSION

This part of the thesis should complement and round-off the discussions at the end of the three results chapter. The mechanism of biased hypermutation and exposure of measles virus RNA to the DRADA enzyme will be discussed first. The role of truncated M protein in measles virus infections is discussed further, along with examples of RNA editing resulting in potential truncation of proteins in other viruses. The possible mechanisms which would result in the insertion of the mitochondrial tRNA are discussed in detail, along with mechanisms which could account for the chimeric YM-V clones isolated. The YM-V cloning results using the general and nested PCR method are discussed and suggestions why the more highly mutated M gene sequence from YM-V passaged in neuroblastoma cell lines was not observed. Finally, further experimentation which could clarify some of the results observed during this project is suggested.

IV.1.1 Biased Hypermutation

The mechanism suggested to account for the biased hypermutation seen within the M gene of SSPE virus material isolated from autopsy brain material involves the action of double-stranded RNA adenosine deaminase - DRADA- previously referred to as double-stranded RNA unwindase/modifying enzyme. The production of U-C transitions observed within the M gene of measles virus is hypothesised to occur by the deamination of adenosine to inosine in both negative and positive sense measles virus RNA (I.2.2.2). This activity was originally localised exclusively in the nucleus of mammalian somatic cells (Wagner *et al.,* 1990), consequently, it is unlikely that under normal conditions measles virus RNA would be a substrate since measles virus replication takes place in the cytoplasm of the cell. However, it has been shown that the production of infectious measles virus particles in enucleated cells does not occur (Follett *et al.*, 1976), thus, it is

possible that under some circumstances the replicative cycle of measles virus may have some dependence on the host cell nucleus since nucleocapsid structures have been observed in the nucleus of infected cells (Dubois-Dalaq *et al.*, 1974b). It is possible that in persistently infected cells the virus genome may be exposed to this enzyme activity at some stage of the cell cycle i.e., mitosis, however, the neuronal cells of the CNS are fully differentiation and, therefore, do not undergo cell division.

It has been shown that there are two forms of the DRADA enzyme; p110, which appears to be located exclusively in the nucleus and is constitutively expressed, and a p150 form which is interferon-inducible and located in both the cytoplasm and the nucleus (Patterson & Samuel, 1995). Type I interferon (α and β) has been shown to be induced in brain cells during persistent measles virus infection, leading to the induction of the MxA protein which actively interferes with measles virus replication (Schneider-Schaulies *et al.*, 1994a). If DRADA is also induced then the cytoplasmic location of this enzyme would allow the measles virus genome to become exposed to this enzyme.

Whether biased hypermutation occurs as a result of the activity of DRADA *in-vivo* remains undetermined. As this enzyme is ubiquitous it seems unlikely that measles virus associated with the CNS is its only target. A-G hypermutation has been described in the provirus of an avian retrovirus (Hajjar & Linial, 1995), vesicular stomatitis virus (O'Hara *et al.*, 1984) and in two escape mutants of huRS virus (Rueda *et al.*, 1994). More recently, RNA editing of hepatitis delta virus (HDV) negative sense RNA (anti-genomic) by DRADA has been observed, resulting in the production of the larger hepatitis delta antigen (HDAg-p27) (Polson *et al.*, 1996). The fact that other RNA viruses have been

shown to exhibit hypermutations, and that DRADA activity does have a functional role in HDV, suggests that this phenomenon may be a naturally occurring event in virus replication.

In measles virus pre-existing hypermutated variants might be positively selected during the development of a persistent infection within the CNS. It has been hypothesised that the DRADA enzyme directs the deamination of adenosine residues in both strands of duplex RNA, suggesting that the U-C transition observed in positive sense mRNA correlates with an A-G transition in the negative sense genome (Bass *et al.*, 1989). However, it should be noted that double-stranded RNA is not an intermediate in measles virus replication, thus double-stranded RNA is only likely to occur as a result of local collapse of mRNA onto the negative strand template during synthesis.

All sequence data from the M gene of SSPE virus has been carried out by cloning cDNA derived from poly A RNA i.e. plus sense mRNA. Confirmation of the A-G transition in the negative sense RNA genome needs to be undertaken to confirm whether hypermutations can occur in both strands of duplex RNA. Alternatively, error prone RNA polymerase complexes could possibly be responsible for biased hypermutation by incorporating U or C residues when copying A residues. Imbalances in the nucleotide pools may also be responsible for hypermutational events. G-A hypermutation in the HIV type I genome has been shown to be related to fluctuations in the intracellular dCTP pool which force the viral reverse transcriptase into making errors (Vartanian *et al.*, 1994).

Biased hypermutated M gene species were not observed in the experiments described in this thesis. This would not be an unexpected result in virus passaged in Vero cells, as the activity of DRADA in this cell line is low (Rataul *et al.*, 1992). However, in the neuroblastoma cell line, IMR-32, the activity of this enzyme was found to be substantially higher. *In-vitro* synthesised 5' sense and anti-sense oligos corresponding to the M RNA from the Edmonston strain of measles virus was found to be a substrate for DRADA from the nuclear (Rataul *et al.*, 1992) and cytoplasmic (Ecker *et al.*, 1995) extracts of IMR-32 cells, also double-stranded M gene fragments and double-stranded RNA covering the entire M gene were found to be substrates for DRADA (Ecker *et al.*, 1995). Therefore, it is surprising that hypermutated M gene was not observed in these experiments. It is possible that a sub-population of M gene species was hypermutated, but due to the procedures used a true representation of the M gene population present was not determined (III.2.4.4). In the experiments reported here the M gene clones isolated from passaged Yamagata-1 virus did not exhibit additional hypermutational events as a consequence of propagation in the neuroblastoma cell lines, which is contrary to the findings of Wong *et al.* (1989). The cloning procedures described (III.3.2 & III.3.7.2) may not have been adequate to reveal a minority component. Ecker *et al.* (1995) found that measles virus sequences already exhibiting hypermutation were less efficiently modified by DRADA *in-vitro,* when compared to the Edmonston strain sequence. Therefore, the presence of more highly mutated M gene sequence in the Yamagata-1 virus population would, theoretically, be more difficult to detect.

IV.1.2 Heterogeneity of Measles Virus RNA

The clones of ADD-MV M gene showed that the 3' region of the gene was deleted. As already stated (III.2.4.4) there are several possible explanations as to how this may have occurred. The recovery of a 800 base fragment when using primers designed to amplify the entire M gene (1.4 kb), suggests that mis-priming may have occurred. However, the sequencing data confirmed the presence of the 3'-end primer (MMO1) binding sequence. This sequenced defined the end of the clone. If the MMO1 primer had been mis-priming one would expect to detect ADD-MV sequence following the primer binding sequence. This was not observed which suggests that these deleted M genes are present in the original ADD-MV stock. Some sequence heterogeneity was observed in the 24 clones sequenced in full. This may reflect polymerase malfunction during amplification of the M gene. Upto 5 base changes, when compared to EdM, were observed in any one clone. Therefore, the error rate of the polymerase when amplifying a 750 base pair fragment would have to be at least 0.66%, a rather high figure. Another possibility is that these different clones may represent pre-existing variants of the measles virus M gene, suggesting that this virus may exist as a quasispecies.

The translation initiation codon of the M gene ORF was intact in all 24 clones, however the stop codon was deleted. Potential stop codons are present down stream from the deletion in all but two of the clones, thus, it is possible that aberrant proteins could be translated. The expression of the aberrant M proteins has not been confirmed. An N gene deletion mutant, Alb4, has been isolated from the coronavirus, mouse hepatitis virus. This mutant contained an 87 nucleotide deletion at the 3'-end of the N gene, however, a

translatable ORF remained intact (Koetzner *et al.*, 1992). Initially, the full length M gene could be amplified, suggesting that an intact M gene is present, albeit at a lower concentration, so that this species of M gene was not readily amplified in these experiments. If the proteins expressed from the deleted clones are functional, it implies that the 3' region of the gene is not essential for the replication of measles virus in cultured cells. Other examples of truncated genes have been reported. A T-A mutation in the H gene of 4 out of 5 measles virus isolates from the Coventry area creates an early termination signal. The predicted H protein would be truncated by 35 amino acids at the C-terminus, which could result in the loss of a neutralising epitope (Outlaw & Pringle, 1995). mRNA editing can result in production of truncated gene products. For example, at the 3'-end of the G protein mRNA of huRS virus there is a run of A residues. Occasionally an A base is deleted or inserted within this region of the gene, producing frame-shifts in the ORF which result in the translation of truncated proteins (Garcia-Barreno *et al.*, 1990; Cane *et al.*, 1993; Dr. J. Evans, University of Warwick, personal communication). As such, mRNA editing resulting in the production of aberrant proteins appears to be a relatively common phenomenon.

The size of the deleted region in the M gene clones varied, and there was some heterogeneity in the M gene sequence. The heterogeneity of the clones of the M gene of ADD-MV indicates that measles virus may exist as a quasispecies. Baczko *et al.* (1993) carried out a study which involved the cloning of measles virus M gene from several regions of the brain of an SSPE patient. The cloning strategy should have resulted in full length M gene clones, however, approximately 80% of the clones sequenced contained deletions of various lengths within the 3' region of the gene. Both wild-type-like

sequence and hypermutated M gene sequence were observed, and *in-situ* hybridisation using probes to both negative and positive sense RNA produced signals, which suggests that both acute virus and 'SSPE virus' are replicating within the brain tissue of SSPE patients. Approximately 15% of the sequences determined were of wild-type-like genes. Positive sense cDNA clones of the M gene of measles virus from two SSPE patients (A and B) were sequenced by Cattaneo *et al.* (1988), patient B being the same patient used in the study carried out by Baczko *et al.* (1993). Some of the M gene clones were found to be incomplete at the 3' end. Deletions ranging from 90 to 632 nucleotides were observed. One of the clones sequenced contained a deletion at nucleotide position 756, which introduced a frame-shift resulting in the production of a stop codon at position 793-795. It was hypothesised that an altered M protein would be translated. Using the rabbit reticulocyte lysate system this deleted clone was translated, and a protein with a apparent molecular weight of 25 kD was detected.

As deleted M gene clones have been detected in both ADD-MV and from viral RNA extracted from brain tissue of an SSPE patient, it seems likely that hypermutation events may not be restricted to full length M gene RNA, but can also occur to the defective M gene population already present. The presence of the deleted M gene species cannot be related to passage history, as the smaller PCR fragment could be generated from both early and late passaged virus. It seems likely that viruses with these defective genes are present in the original virus stock. However, the concentration of the defective genomes may have increased during *in-vitro* passage of the virus. The virus titre of ADD-MV passaged in Vero cells, a cell line highly permissive for measles virus propagation, did diminish during passage. This may reflect an increase in the relative concentration of

defective interfering particles. Alternatively, Lecouturier *et al.* (1996) have suggested that multiple virus passage in Vero cells selects for measles virus isolates with affinity for CD46, the putative measles virus receptor. Measles virus infection via the CD46 receptor molecule leads to down-regulation of the receptor, which may result in attachment and entry of fewer virus particles and a fall in virus titre.

It is possible that the deleted M gene was amplified from measles virus particles with defective genomes within the virus population. The high proportion of deleted M genes is not consistent with the moderated level of infectivity observed. DI particles have been suggested to play an important role in development of persistent infections. However, there was no obvious reduction in virus titre over the 10 passages of ADD-MV in each of the four cell lines which suggests that the phenotype of the virus was not changed. If the concentration of DI particles was high one might have expected that the infection would develop to a persistent phenotype. To determine whether DI particles were present in high concentration the virus preparations should be examined by limiting dilution infections. If DI particles are present, diluting the inoculum will reduce the concentration, and at a dilution where there is minimal interference from DI particles the titre of that virus would be expected to increase.

To investigate the deleted M gene products further, they could be sub-cloned into expression systems, and the resulting protein purified and detected via western blot using either polyclonal measles virus antisera or monoclonal antisera that was raised to an epitope within the amino terminal region of the M protein. The ability of the protein expressed from the deleted M gene clones to interact with the nucleocapsid and, possibly

the membrane associated proteins could also be explored. However, as the 3'-end of the mRNA is deleted is unlikely that the translated protein would interact with the F and H proteins as it is the carboxy terminus that is thought to be responsible for this interaction.

IV.1.3 Evidence of Recombination in Measles Virus

A 54 base insertion has been reported in the HA gene of influenza virus, a segmented, negative-sense RNA (Khatchikian *et al.*, 1989). The inserted nucleotide sequence resembled a region of the 28S ribosomal RNA. The suggested mechanism for this type of recombination was that the rRNA was used as a template during the synthesis of positive-stranded viral RNA from the negative-sense template by polymerase jumping. Recombination has been reported for most positive strand RNA viruses and non-homologous recombination is thought to be responsible for the presence of a modified cellular $tRNA^{Asp}$ at the 5' terminus of naturally-occurring DI RNA's of Sindbis virus (Monroe & Schlefinger, 1983). The cellular gene sequence ubiquitin has been shown to be integrated into the positive sense RNA genome of bovine viral diarrhoea virus (BVDV) (Meyers *et al.*, 1991). The absence of recombination in non-segmented negative sense RNA viruses is thought to be a consequence of the encapsidation of replicative RNA.

In 13 of the 24 ADD-MV clones sequenced in this project, a 50 base insertion was observed. This sequence has high percentage identity with human mitochondrial Leu transfer RNA ($tRNA^{Leu}$). Whether non-homologous recombination could occur between viral RNA and mitochondrial tRNA is unknown. Mitochondria are capable of DNA

replication, mRNA synthesis and protein synthesis. However, mitochondrial RNA is not usually present in the cell cytoplasm which is the site of measles virus replication. Two 5'- and 3'-endonucleases (mitochondrial RNase P and nuclear RNase P) and an ATP(CTP) nucleotidyltransferase are required to process human mitochondrial tRNA precursors (Rossmanith *et al.*, 1995). Both mitochondrial and nuclear RNase P recognise (mt)pre-$tRNA^{Leu}$ as a substrate. However, (mt)pre-$tRNA^{Tyr}$ is cleaved by mitochondrial RNase P, but is not a substrate for nuclear RNase P. (mt)pre-$tRNA^{Leu}$ is the most conventional mitochondrial tRNA in that it retains many of the nucleotides that are conserved in non-organelle tRNAs (Anderson *et al.*, 1981; Kim *et al.*, 1974). Its tertiary structure may, therefore, be more similar to cytoplasmic tRNAs than that of other mitochondrial tRNAs. The 50 base insertion may, therefore, be a cytoplasmic tRNA that has been processed by the nuclear RNase P. This would be located in the cytoplasm although why it should recombine with measles virus RNA during viral replication is unclear.

Recombination by a cross-over event requires two areas of homology between the sequence that is integrated and the target sequence. Once recombination has taken place there is no obvious homology at the site of integration. No homology is observed at the site of integration of the $tRNA^{Leu}$ and the M gene of measles virus, suggesting that non-homologous recombination may have taken place. If transpositional recombination had occurred short repeats of target sequence i.e., measles virus M gene sequence, would be observed surrounding the integrated sequence. This method of recombination is mediated by an integrase enzyme, which would have to be encoded by the mitochondrial DNA as the material to be integrated is of mitochondrial origin. Mitochondria are thought to have evolved from endocytosed bacteria, and many of the genes encoding

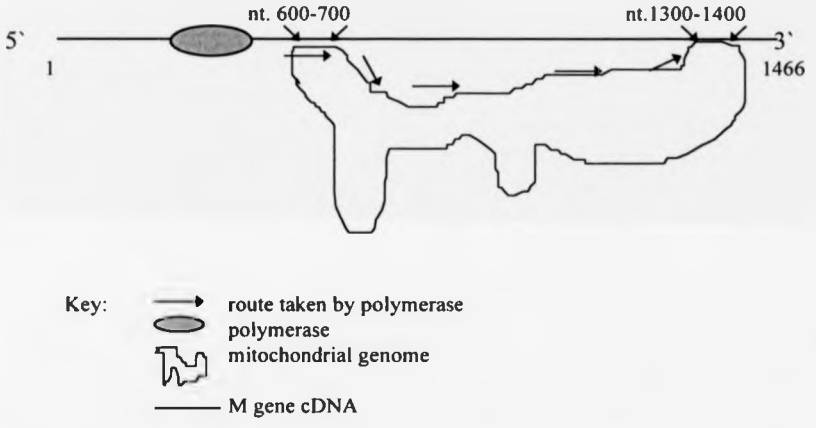
mitochondrial proteins are located in the cell nucleus, which suggests that extensive gene transfers from organelle to the cell nucleus had occurred. However, the sequence of the genome of human mitochondria has been determined and sequence encoding an integrase enzyme has not been found. Integrases are present within cells, although they do tend to be specific for a particular transposon, therefore, it is unlikely that this method of recombination was responsible for the generation of the clones containing the 50 base insertion. The fact that the inserted sequence was found in the same region of the M gene in each clone is probably a consequence of the experimental protocol, namely, the clones were generated from PCR products, so each clone may have been generated from the same cDNA species. The slight variation in the sequence of the clones may represent polymerase errors during the amplification procedure.

The possibility that the 50 base insertion observed in the M gene clones of ADD-MV was a PCR artefact can not be entirely dismissed. If regions of homology between ADD-MV M gene and mitochondrial genome flanked the mitochondrial $tRNA^{Leu}$ sequence and the mitochondrial genome was positioned close to the M gene of ADD-MV at these regions, it is possible that the polymerase could slip from the M gene of ADD-MV to the mitochondrial genome (Figure 51). At another region of homology downstream the polymerase could then slip back onto the M gene which would result in the insertion of the tRNA.

This explanation could also account for the observation of deleted M gene clones. Rather than the polymerase slipping onto the mitochondrial genome, the region of the M gene not homologous to the mitochondrial sequence could loop-out, and the polymerase could

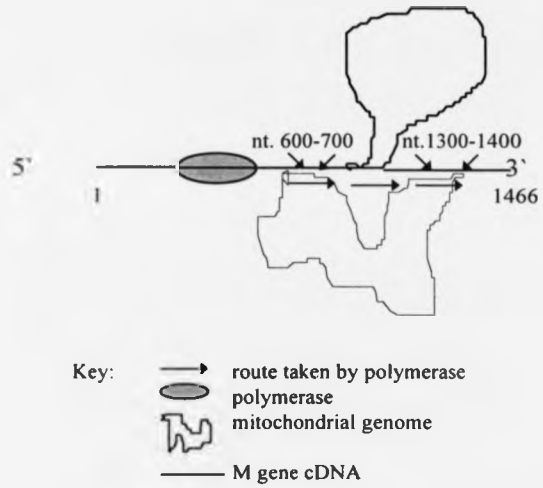
slip across the junction, resulting in an apparent deletion in the M gene (Figure 52). Homology between the M gene of measles virus and the mitochondrial genome may not be necessary for such a loop to occur. Regions of homology within the M gene of measles virus itself may base pair, resulting in the formation of a loop, which the polymerase could jump across, producing a deletion within the gene.

Figure 51. Possible mechanism for the 50 base insertion by PCR artefact



The entire length of mt-tRNA^{Leu} is only 75 nucleotides and the inserted sequence is 50 nucleotides, therefore, if homology was occurring between the M gene and mitochondrial sequence, it would have to be somewhere else within the mitochondrial genome. To determine if this was indeed occurring regions of identity between the M gene of ADD-MV (accession number: U01981) and the human mitochondrial genome (accession number: J01415) were searched for using the Genetics Computer Group (GCG) program.

Figure 52. Possible mechanism for M gene deletions by PCR artefact



Only one region of identity was found, this corresponded with the M gene at nucleotide position 246-277, and the mitochondrial genome at 1266-1297. Within this region there was a 71.9% identity. The position of the $tRNA^{Leu}$ within the mitochondrial genome is at nucleotides 3231-3305, and the insertion in the M gene of ADD-MV occurred at nucleotide position 671-1348. If the insertion had occurred as consequence of PCR error, there would have to be homology at or around nucleotide positions 670 and 1340 on the M gene of ADD-MV, and, 3200 and 3300 on the mitochondrial genome. This was not found. In order to full investigate the possibility of PCR error, identity between the M gene of measles virus and the mitochondrial genome surrounding the $tRNA^{Leu}$ were determined. A 40 nucleotide base sequence 5' to the $tRNA^{Leu}$ was found to have some identity with the M gene at nucleotide position 384-401. Within this 18 base region 14 (77.8%) bases were identical. However, the position within the M gene were the identity was found does not correlate with the position of the inserted sequence. A 30 nucleotide

sequence 3' to the $tRNA^{Leu}$ was also used to search for identity with the M gene of measles virus. An 11 base region of the 30 base query sequence contained 90.9% identity with the M gene, however, the position within the M gene where this occurred was 383-393, not were the inserted sequence occurred. It, therefore seems unlikely that the insertion occurred as a consequence of PCR error.

The sequence of two chimeric clones derived from YM-V also suggests that recombination may occur rarely, possibly as a consequence of a delay or failure in encapsidation of replicative RNA. One M gene clone (III.3.7.4) from Yamagata-1 virus passaged 5 times in IMR-32 cells contained sequence corresponding to the M gene of Yamagata-1 virus (Wong *et al.*, 1989) up to position 457 (G-A) after this base change the sequence reverted to that of EdM (Bellini *et al.*, 1986). An M gene clone of YM-V propagated in MRC-5 cells followed by 5 passages in IMR-32 cells (III.3.7.6) contained EdM-like sequence up to position 457, after this base change the sequence reverted to that of the M gene of Yamagata-1 virus (Wong *et al.*, 1989). This could be an example of homologous recombination between the lytic measles virus genome and the cell-associated Yamagata-1 virus. Homologous recombination involves the exchange of two comparable RNA regions at precise locations. The site of the recombination between these two virus populations appears to be position 457. The G-A base change at position 457 is the only nucleotide substitution which is common to both the M gene sequence of ADD-MV passaged once and 10 times in all four cell lines used in these experiments and the Yamagata-1 virus M gene sequence determined by Wong *et al.*, 1989. These data suggest that homologous recombination between negative-sense RNA viruses is possible. Template switching by the polymerase is a possible explanation for the generation of

these clones. However, it should also be considered that these recombinant M gene sequences may have been amplified from DI genomes.

IV.1.4 Yamagata-1 Virus - a Heterogeneous Population

The initial conclusion made from the sequencing data of the YM-V clones was that the virus population was heterogeneous. The EdM sequence was observed along with the M gene sequence of Yamagata-1 virus determined by Wong *et al.* (1989). These findings are similar to those of Baczko *et al.* (1993) who determined, using autopsy material from an SSPE patient (B), that hypermutated measles virus expanded clonally from several sites throughout the brain, but differs from the results of Wong *et al.* (1989), who found that passage of the Yamagata-1 virus in the neuroblastoma cell line resulted in the production of additional mutation within the M gene when compared to virus passaged in Vero cells only. In that study wild-type M gene sequence was not detected, however, the number of clones analysed was much fewer than that done in the experiments detailed in this thesis, or the study carried out by Baczko *et al.* (1993).

The wild-type-like sequence (group B1) described by Baczko *et al.* (1993) contained 5 base changes when compared to the most closely related acute disease measles virus M gene sequence. Group B2 contained sequence similar to that of B1, except that 13 U residues were replaced by Cs. This virus group contained mutated M genes but the translation initiation codon was intact and so the M protein could be translated. The M gene fragments of YM-V cloned into the vector pGEMT (III.3.7) in this project were similar to that of group B2, determined by Baczko *et al.* (1993). These clones did contain

a mutated M gene sequence when compared to EdM, but the translation initiation codon was intact, so a full length protein could be produced. Hypermutated M gene clones from groups B3, B4 and B5, determined by Baczko *et al.* (1993) contained a mutation which destroyed the translation initiation codon. The M gene fragments of YM-V, which resembled the M gene of Yamagata-1 virus (Wong *et al.,* 1989), cloned in the bacteriophage M13 also contained a mutation which destroyed the translation initiation codon. Although their was no evidence of expansion of the degree of hypermutation within the clones isolated from passaged YM-V in this project, M gene clones were isolated which contain both intact and non-functional translation initiation codons, so *in-vitro* isolated clones, to some extent, did follow a similar pattern to those isolated from *in-vivo* i.e., isolated from autopsy material.

As the study of Baczko *et al.* (1993) has isolated both wild-type-like M gene clones and hypermutated M gene clones, it seems unlikely that the EdM clones isolated in this project, from the YM-V passaged material, represent a contaminating virus. The majority of the clones (80%) isolated by Baczko *et al.* (1993) contained deletions within the 3'-end of the gene. Although such deleted M gene fragments were detected from passaged ADD-MV, none were isolated from the passaged YM-V. This is an unusual result in that one might expect to detect more defective genes from virus with a persistent phenotype as DI particles are predicted to have a role in the development of persistence (Huang & Baltimore, 1970). As acute virus (or wild-type-like) M gene clones were detected from the brain material of patient B (Baczko, *et al.,* 1989) resembled that of the measles virus strain JB, it is possible that this strain of measles virus was the initial infective agent and that deleted M genes were present within the virus population. These deletion mutants

appear to have also undergone hypermutation. The progenitor strain of Yamagata-1 virus is not known. It is possible, therefore, that the acute disease progenitor of Yamagata-1 virus does not produce deleted M gene species during an acute infection. If this was the case one would not expect to isolate such species from the mutated virus. However, the primer (YM2) used to amplify the Yamagata-1 virus M gene is positioned within the region of the M gene which was deleted from the ADD-MV population. It is unlikely, therefore, that deleted M gene would be amplified using this primer. To determine whether a sub-population of deleted M gene is present, the amplification procedures would need to be repeated using a primer which would anneal at the 3' end of the gene.

The percentage of EdM sequence and YM-V sequence was compared between the two methods of M gene amplification i.e., traditional PCR and nested PCR. The nested PCR results correlated with the results from the study of Baczko *et al.* (1993). Approximately 16% of the clones obtained using nested PCR were of the EdM sequence compared with the 15% reported by Baczko *et al.* (1993). However, for the traditional PCR method i.e., using only one set of PCR primers, 60% of the clones produced sequence corresponding to the EdM sequence. This could reflect the specificity of the PCR reaction. The nested PCR method is more specific and sensitive, so may reflect more accurately the proportion of wild-type : mutated M gene species present within the SSPE virus population.

As both acute virus and 'SSPE virus' M gene could be detected, the experiment could be repeated using plaque purified YM-V. The resulting virus clone would need to be sequenced to determine whether the M gene sequence was that of YM-V or the acute virus. The purified YM-V could then be passaged and the cDNA cloned in a library

rather than the amplified M gene being isolated by PCR. This would give a better representation of the population of M gene sequences within the virus population.

IV.1.5 Future Research Priorities

IV.1.5.1 Deleted M Gene Population

The presence of the deleted M gene population in the original virus stock needs to be confirmed as it is possible that the clones isolated from the experiments detailed here were produced due to the formation of unique ER sites by base substitutions. This could be done by making a cDNA library and isolating M gene clones using oligonucleotide probes relating to the 5'-end of the M gene cDNA. If deleted M gene clones are isolated this would suggest that the clones isolated in this project were not artefacts of the amplification and/or cloning procedures.

To determine whether functional protein could be translated from the deleted M gene population, the clones would have to be subcloned into an expression vector and the resulting protein purified. This protein could be analysed by western blot using polyclonal sera and/or monoclonal sera raised to epitopes at the amino terminus of the protein to determine the apparent molecular weight of the protein. The ability of the protein to interact with the nucleocapsid could be determined. The carboxy terminus is thought to be involved in the interaction of M protein with the surface glycoproteins (F and H), therefore it is unlikely that the deleted clones would still maintain this function. Reverse genetics of measles virus has been reported (Radecke *et al.*, 1995). This system could be employed using the deleted M gene in the mini-genome, rather than the full

length, to determine what effects, if any, the deleted M gene may have on the phenotype of the infection. As the interaction between the M protein and the F and H proteins would not be expected to occur, one could hypothesise that assembly of the virus would be destroyed and a persistent infection would ensue.

IV.1.5.2 Yamagata-1 Virus Hypermutation and Recombination

The first priority would be to make a cDNA library and to isolate M gene clones using oligonucleotide probes to the M gene of Yamagata-1 virus (Wong *et al.*, 1989). A large number of clones would have to be isolated and sequenced from virus passaged in IMR-32 and SK-N-SH cells to determine whether the M gene population does contain a small population which is more extensively mutated than virus passaged only in Vero cells. If a range of more or less mutated M gene clones was isolated, this would suggest that the clone from Yamagata-1 virus passaged in IMR-32 cells (Wong *et al.*, 1989), which contained 38 additional U-C transitions, was a pre-existing variant rather than a response to propagation in the neuroblastoma cell line.

The sequence of the genomic (- sense) RNA of the Yamagata-1 virus also needs to be determined. As the predicted method of biased hypermutation relies on the deamination of adenosine residues in both strands of duplex RNA it is important that the base changes observed in the mRNA are also present in the genomic material. If this is not confirmed another hypothesis for the development of biased hypermutation would have to be suggested, for example, alkylating damage. RNA polymerase will incorporate cytosine quite readily if adenosine is methylated (1-MeA) (Saffhill *et al.*, 1985).

Investigations to determine whether the chimeric clones of YM-V were the product of homologous recombination involving breaking and rejoining strands at regions of homology, or a copy choice mechanism in which the viral RNA polymerase switches templates during replication should be carried out. Using the reverse genetics, M gene sequence that does not contain the A-G base change at position 457 could be cloned into the mini-genome and the resulting virus progeny co-infected with plaque-purified Yamagata-1 virus. The Yamagata-1 virus stock has been shown to contain a heterogeneous population of both acute and mutated M genes. The purified stock would have to contain only the mutated M gene. The resulting virus progeny would be plaque-purified and individual virus plaques propagated to produce a virus stock. The M genes from these stocks would need to be sequenced to determine whether any chimeric clones had been produced. The experiment would also need to be repeated using M gene sequence which does contain the A-G mutation at position 457. An alternative method for determining whether recombination had taken place would be to carry out RNAses A protection assays. A radioactive probe complementary to the EdM sequence around nucleotide position 457 would protect acute M gene, but at the recombination site mis-matches would occur where the sequence corresponds with the Yamagata-1 virus and RNAses A would degrade this region producing a different banding pattern.

APPENDIX A

Attempted Identification of the Unknown Sequence

The unidentified sequence (Figure 53) was isolated from YM-V passaged once and five times in IMR-32 cells, passaged in MRC-5 prior to five passages in SK-N-SH cells and passaged in MRC-5 cells followed by 5 passages in IMR-32 cells.

Figure 53. Comparison of the 5 -end sequence of the unknown PCR fragment and the M gene sequence of measles virus

```
EdM   UACAGCUCAACUUACCUGCCAACCCCAUGCCAGUCGACCAACUAGUACAA    50
Unk   UACAGCUCAACUUACCUGCCCAAAGUGCCCCUCACUUCCCUCCGGCAGAU
      ********************* *       **   *  **  *  * * *

EdM   CCUAAAUCCAUUAUAAAAAACUUAGGAGCAAAGUGAUUGCCUCCCAAGUU    100
Unk   UGUGCUUCUGGAAAGUUGUGUUUAGUAAUAUCUUAAACACUCAUUAUUCA
        *   **    *         **** *  *   * *   *     *

EdM   CCACAAUGACAGAGAUCUACGACUUCGACAAGUCGGCAUGGGACAUCAAA    150
Unk   AGGAACCCUGCGGUGGUUCCUUCUGUAAAAGCAAGGCUCUGUUGGUGAAG
        *             *    **   * *    ***   *    * **

EdM   GGGUCGAUCGCUCCGAUACAACCCACCACCUACAGUGAUGGCAGGCUGGU    200
Unk   GAAAUAACAAGCUCUAAAUGACUCGUAAUAAAUUAGAGCUUUGAUAUAAA
      *     *       * * *  ** *   *   *               *

EdM   GCCCCAGGUCAGAGUCAUAGAUCCUGGUCUAGGCGACAGGAAGGAUGAAU    250
Unk   AAGCUCUGUCCUACAUGUAGGACUUACACGUUAGACGAAUUCUCUUCACC
        *   ***  *    ***  * *   *         *       * *
```

The sequence identity between the 5'-end of the M gene of the Edmonston strain of measles virus (EdM) (Bellini *et al.*, 1986) and the unidentified sequence (unk) produced from Yamagata-1 virus passaged once in IMR-32 cells. The fragment was amplified using the YMP/YM2 primer pair and subsequently cloned into the bacteriophage M13. Sequencing reaction was carried out using the -40 primer, following the manufacturer's protocol for Sequenase sequencing kit (USB). * indicated homologous bases. **Bases in bold-type represent the YMP primer sequence.** *AUG*: translation initiation codon for the M gene of EdM

Identification of the unknown sequence cloned from Yamagata-1 viral passage material was attempted using two approaches. The first method was to carry out a homology search using the Blast Database. Searches carried out upto January 1995 did not reveal any homology with the 666, 201 nucleotide sequences stored on the database. The second approach was to look for measles virus-specific sequence linked to the unknown sequence using PCR primers located in the measles virus genes adjacent to the M gene i.e., the P and F genes.

Only the 5'-end 250 bases of the unknown sequence had been determined. In order to determine whether this M primer amplified product was located between the P and F genes, it was necessary to determine the 3' end of this sequence. The unknown sequence was excised from M13mp18 vector and re-cloned into M13mp19. This reverses the orientation of the insert, so sequence reactions using the -40 primer will produce the 3'-end sequence of the unknown material (Figure 54).

The position of annealing of the 3' primer (YM2) sequence, which was used to amplify the unknown material, could be determined from the sequencing gels, therefore, the sequence obtained was confirmed as the 3' region of the fragment that was originally amplified by PCR and cloned into M13 mp18. A 5' primer (YM4) for further amplification experiments could now be designed.

Figure 54. The 3'-end sequence of the unknown PCR fragment

```
ATACGTATAATGTAAATTGTTCCCTAAGCAAGGCTGCCTATATAATCTAC  812

AAGATAAAAACTGGAAGTGGTTTTCTCTAGCGAGGGTGGCGGGGCTGTCC  862

CTGCCCCACAGGAATCAGGATAACTGCCCTGCCGTGTGTTCAGCACTTTA  912

GAATTTTCACATTCTTCAGAATCCCCTAGCACGGTAGGTAGGCAGTGCTG  962

                                  Sal I
GGACCATTGTCCGCATTTACGACGACGTGTCGACT                 1007
```

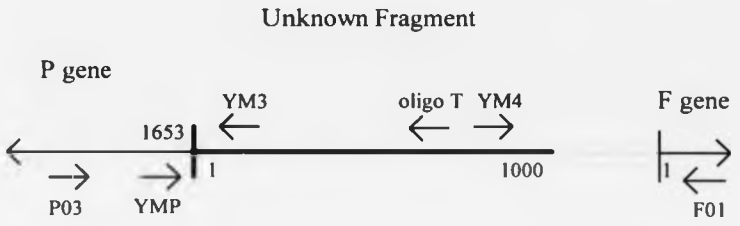
YM2 primer

A total of 6 primers (Table 32 and Figure 55) were used in order to determine if the unknown material, amplified using the primer pair YMP and YM2, was flanked by measles virus P and F genes

Table 32. Primers used for the identification of the unknown fragment

Gene	Name	Position	Sequence	Restriction sites	Tm °C
P	P03	1174 - 1192	5' CGC CAT TCC TGG ACT TGG G 3'	Sal I	62
F	F01	289-273	3' GGC CAG GGT GGT CTG GG 5'	Pst I	60
Unknown	YM3	120 - 102	3' GGA ACC CTG CGG AGG TTC C 5'	Pst I	64
Unknown	YM4	949-967	5' GGT AGG CAG TGC TGG GAC C 3'	Bam HI	64
M	YMP	-73 to -54	5' TAC AGC TCA ACT TAC CTG CC 3'	Hind III	60
poly A tail	Oligo dT	-	5' TTT TTT TTT TTT TTT 3'	Hind III	60

Figure 55. Position of primers for identification of unknown sequence



The following primer pairs were used for amplification experiments, Oligo T and YMP, P03 and YM3, F01 and YM4, YMP and F01 and P03 and F01. For all reactions the same cycle parameters were used, 94°C for 45 secs, 60°C for 45 secs and 74°C for 2 mins for a total of 30 cycles. Table 33 shows the fragment size which should be amplified for the oligo T/YMP and PO3/YM3 primer pairs. For the remaining primer pairs used to identify the unknown sequence, namely, FO1/YM4, YMP/FO1 and PO3/FO3, the expected size of the amplified fragment can not be determined as the full length of the unknown sequence has not been determined.

Table 33. Fragment sizes expected to be amplified if the unknown sequence is linked to measles virus specific gene

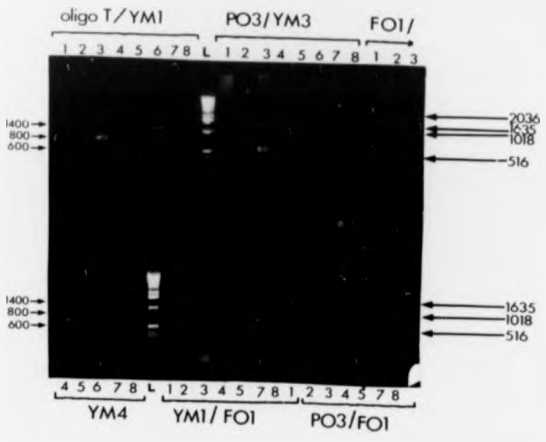
Primer Pair	Expected Fragment Size (bases)
Oligo T and YMP	750
P03 and YM3	584

Each primer pair was used in 8 reactions, with a negative control which did not contain any cDNA. Mock infected IMR-32 cDNA was used to verify that the primers did not bind to cellular material. ADD-MV cDNA, synthesised from total RNA extracted from infected Vero cells, was also used as a control. PO3, YMP, FO4 and oligo T would be

expected to anneal to ADD-MV material. However, primers designed from the unknown sequence i.e., YM3 and YM4, will not be expected to bind, therefore, for these reactions the ADD-MV cDNA should be acting as a negative control. Yamagata-1 virus passaged in MRC-5 cells and once in IMR-32 cells cDNA was also used as the clones obtained from this passaged virus (III.3.5.4) resembled the M gene of YM-V (III.3.4), again, the YM3 and YM4 primers would not be expected to bind to this material. And finally the four cDNA's that correspond to the virus passage that produced only this unknown sequence were examined; i.e., Yamagata-1 virus passaged once and 5 times in IMR-32 cells; Yamagata-1 virus passaged in MRC-5 cells followed by 5 passages in IMR-32 cells; Yamagata-1 virus passaged in MRC-5 cells and 5 times in SK-N-SH cells. Figure 56 shows the results of these PCR reactions.

Amplifications using cDNA synthesised from ADD-MV resulted in the amplification of fragments even when primers designed from the unknown material were used, i.e., PO3/YM3 primer pair (Figure 56). The fact that these primers did anneal to ADD-MV material made it impossible to confirm whether the unknown material was linked to the P and F genes of measles virus. It is possible that bicistronic transcripts were not synthesised at a detectable frequency in the material used in this experiment. Re-designing the experiment so that genomic material would be amplified may be required to determine whether measles virus genes are associated with the unknown material. Another approach would be to use this unknown material as a probe and carry out northern blots on RNA isolated from Yamagata-1 virus infected cells, ADD-MV infected cells and uninfected cells to determine whether this material does bind to measles virus RNA species. As the 3` and 5`-ends of this unknown material are known, the full length

Figure 56. PCR reactions with the primers designed to identify the unknown fragment



1: negative control; 2: mock infected IMR-32 cells; 3: ADD-MV; 4: virus grown in MRC-5 cells and one passaged in IMR-32 cells; 5: virus grown in MRC-5 cells and 5 passages in IMR-32 cells; 6: virus passaged once in IMR-32 cells; 7: virus passaged 5 times in IMR-32 cells; 8: virus grown in MRC-5 cells and 5 passages in IMR-32 cells. Total RNA was isolated from these virus passages, cDNA was amplified using Oligo T, as such, bicistronic mRNA would be the material amplified in these experiments. SSPE brain material has been shown to contain a high proportion of bicistronic message (Cattaneo *et al.*, 1986; Hummel *et al.*, 1994). The cycle used was 94°C for 45 secs, 60°C for 45 secs and 74°C for 2 mins (30 cycles). 10 µl of the reaction was run on a 1% agarose TBE gel.

of this unknown material could be sequenced and then used to search the database again, to determine if any homology exists between this material and any of the nucleotide sequences stored on the database.

BIBLIOGRAPHY

Aarli, J.A. (1974): Nervous complications of measles: clinical manifestations and prognosis. *Eur. Neurol.* **12**, 79-93.

Aicardi, J., Goutieres, F., Aresnio-Nunes, M.L. and Lebon, P. (1977): Acute measles encephalitis in children with immunosuppression. *Paediatr.* **59**, 232-239.

Albtrect, P. and Ennis, F.A. (1977): Persistence of maturnal antibody in infants beyond 12 months: mechanism of measles vaccine failure. *J. Paediatr.* **91**, 175-178.

Alkhatib, G., Richardson, C. and Shen, S-H. (1990): Intracellular processing, glycosylation and cell-surface expression of the measles virus fusion (F) protein encoded by a recombinant adenovirus. *Virol.* **175**, 262-270.

Alkhatib, G., Roder, J., Richardson, C., Briedis, D., Weinberg, R., Smith, D., Taylor, J., Paoletti, E. and Shen, S-H. (1994a): Characterisation of a cleavage mutant of the measles virus fusion protein defective in syncytium formation. *J. Virol.* **68**, 6770-6774.

Alkhatib, G., Shen, S-H., Briedis, D., Richardson, C., Massie, B., Weinberg, R., Smith, D., Taylor, J., Paoletti, E. and Roder, J. (1994b): Functional analysis of N-linked glycosylation mutants of the measles virus fusion protein synthesised by recombinant vaccinia virus vectors. *J. Virol.* **68**, 1522-1531.

Anderson, S., Bankier, A.T., Barrell, B.S., de Bruijn, M.H.L., Coulsen, A.R., Drouin, J., Eperson, I.C., Nierlich, D.P., Roe, B.A., Sanger, F., Schreier, P.H., Smith, A.J.H., Staden, R. and Young, I.G. (1991): Sequence and organisation of the human mitochondrial genome. *Nature.* **290**, 456-465.

Arnheiter, H., Davis, N.L., Wertz, G., Schubert, M. and Lazzarini, R.A. (1985): Role of nucleocapsid protein in regulating VSV RNA synthesis. *Cell.* **41**, 259-267.

Ayata, M., Hirano, A. and Wong, T.C. (1991): Altered translation of the matrix genes in Niigata and Yamagata neurovirulent measles virus strains. *Virol.* **180**, 166-174.

Baczko, K., Liebert, U.G., Billeter, M.A., Cattaneo, R., Budka, H. and ter Meulen, V. (1986): Expression of defective measles virus genes in brain tissues of patients with SSPE. *J. Virol.* **59**, 472-478.

Baczko, K., Brinckmann, U., Pardowitz, I., Rima, B.K. and ter Meulen, V. (1991): Nucleotide sequence of the genes encoding the matrix protein of two wild-type measles virus strains. *J. Gen. Virol.* **72**, 2279-2282.

Baczko, K., Pardowitz, J., Rima, B.K. and ter Meulen, V. (1992): Constant and variable regions of measles virus proteins encoded by the nucleocapsid and phosphoprotein genes derived from lytic and persistent infections. *Virol.* **190**, 496-474.

Baczko, K., Lampe, J., Liebert, U.G., Brinckmann, U., ter Meulen, V., Pardowitz, I., Budka, H., Cosby, S.L., Issorte, S. and Rima, B.K. (1993): Clonal expansion of hypermutated measles virus in SSPE brain. *Virol.* **197**, 188-195.

Ballart, I., Huber, M., Schmid, A., Cattaneo, R. and Billeter, M.A. (1991): Functional and non-functional measles virus matrix genes from lethal human brain infection. *J. Virol.* **65**, 3161-3166.

Banerjee, A.K. (1987): Transcription and replication of Rhabdoviruses. *Microbiol. Rev.* **51**, 66-87.

Banerjee, A.K. and Barik, S. (1992): Gene expression of vesicular stomatitis virus genomic RNA. *Virol.* **188**, 417-428.

Bankamp, B. Brinckman, U.G., Reich, A., Niewiesk, S., ter Meulen, V. and Liebert, M.G. (1991): Measles virus nucleocapsid protein protects rats from encephalitis. *J. Virol.* **65**, 1695-1700.

Barr, J., Chambers, P., Pringle, C.R. and Easton, A.J. (1991): Sequence of the major nucleocapsid protein gene of pneumovirus of mice, sequence comparisons suggest structural homology between nucleocapsid proteins of pneumoviruses, paramyxoviruses, rhabdoviruses and filoviruses. *J. Gen. Virol.* **72**, 676-685.

Barrett, T., Shrimpton, S.B. and Russel, S.E.H. (1985): Nucleotide sequence of the entire protein coding region of canine distemper virus polymerase-associated (P) protein mRNA. *Virus Res.* **3**, 367-372.

Barrett, T. and Underwood, J. (1985): Comparisons of mRNAs induced in cells infected with each member of the morbillivirus group. *Virol.* **145**, 195-199.

Barrett, T., Clark, D.K., Evans, S.A. and Rima, B.K. (1987): The nucleotide sequence of the gene encoding the fusion protein of canine distemper virus: a comparison of the deduced amino acid sequence with the other paramyxoviruses. *Virus Res.* **8**, 373-376.

Barrett, T, Subbarao, S.M., Belsham, G.J. and Mahy B.W.J. (1991): The molecular biology of the morbilliviruses. In: Kingsbury, D.W. (Ed). *The paramyxoviruses.* Plennum Press, New York. 83-102.

Bartlet, M.S. (1957): Measles periodicity and community size. *JR.Stat. Soc. A.* **120**, 48-70.

Bass, B.L. and Weintraub, H. (1987): A developmentally regulated activity that unwinds RNA duplexes. *Cell.* **48**, 607-613.

Bass, B.L., Weintraub, H., Cattaneo, R. and Billeter, M.A. (1989): Biased hypermutation of viral RNA genomes could be due to unwinding / modification of double-stranded RNA. *Cell.* **56**, 331.

Bech, V. (1959): Studies on the development of complement fixing antibodies in Measles patients. *Immunol.* 83, 267-275.

Beckford, A.P., Kaschula, R.O.C. and Stephen, C. (1985): Factors associated with fatal cases of measles: a retrospective autopsy study. *S. Afr. Med. J.* **68**, 858-863.

Bellini, W.J., McFarlin, D.E., Silver, G.D., Mingiolo, E.S. and McFlarland, H.F. (1981): Immune reactivity of purified haemagglutinin of measles virus. *Infect. Immunol.* **32**, 1051-1057.

Bellini, W.J., Englund, G., Rozenblatt, S., Arnheiter, H. and Richarson, C.D. (1985): Measles virus phosphoprotein gene codes for two proteins. *J. Virol.* **53**, 908-919.

Bellini, W.J., Englund, G., Richardson, C.D., Rozenblatt, S. and Lazzarini, R.A. (1986): Matrix genes of measles virus and canine distemper virus: cloning, nucleotide sequence and deduced amino acid sequence. *J. Virol.* **58**, 408-416.

Billeter, M.A., Baczko, K., Schmid, A. and ter Meulen, V. (1984): Cloning of DNA corresponding to four different measles virus genomic regions. *Virol.* **132**, 147-159.

Billeter, M.A., Cattaneo, R., Speilhofer, P., Kaelin, K., Huber, M., Schmid, A., Baczko, K. and ter Meulen, V. (1993): Generation and properties of measles virus mutations typically associated with SSPE. *Ann. N.Y. Acad. Sci.* 1-21.

Black, F.L. (1966): Measles endemicity in insular populations: critical community size and its implications. *J. Theor. Biol.* **11**, 207-211.

Black, F.L., Berman, L.L., Borgono, K.M., Capper, R.A., Carvalhol, A.A., Collins, C., Glover, O., Hajazi, Z., Jacobson, D.L., Lee, Y.L. *et al.*. (1986): Geographic variation in infant loss of maternal measles virus antibodies and in prevalence of rubella antibodies. *Am. J. Epidemiol.* **124**, 442-452.

Black, F.L. (1989): Measles active and passive immunity in a worldwide prospective. *Prog. Med. Virol.* **36**, 1-33.

Blau, D.M. and Compans, R.W. (1995): Entry and release of measles virus are polarised in epithelial cells. *Virol.* **210**, 91-99.

Blumberg, B.M. and Kolakofsky, D. (1981a): Intracellular VSV leader RNAs are found in nucleocapsid structures. *J. Virol.* **40**, 568-576.

Blumberg, B.M., Leppert M. and Kolakofsky, D. (1981b): Interaction of VSV leader RNA and nucleocapsid protein may control VSV genome replication. *Cell.* **23**, 837-845.

Blumberg B.M., Giorgi, C. and Kolakofsky, D. (1983): Nucleocapsid protein of VSV selectively encapsidates leader RNA *in-vitro*. *Cell.* **32**, 559-567.

Blumberg, B.M., Giorgi, C., Roux, L., Dowling, P., Chollet, A. and Kolakofsky, D. (1985): Sequence determination of the Sendai virus HN gene and its comparison to the influenza virus glycoproteins. *Cell.* **41**, 269-278.

Blumberg, B.M., Crowley, J.C., Silverman, J.I., Menonna, J., Cook, S.D. and Dowling, P.C. (1988): Measles virus L protein: evidence of elements of ancestral RNA polymerase. *Virol.* **164**, 487-497.

Blumberg, B.M., Chan, J. and Udem, S.A. (1991): Function of paramyxovirus 3' and 5' end sequences in theory and practice. In: Kingsbury, D.W. (Ed). *The paramyxoviruses*. Plennum Press, New York. 235-247.

Bohn, W., Rutter, G., Hohenberg, H. and Mannweiler, K. (1983): Inhibition of measles virus budding by phenothiazines. *Virol.* **130**, 44-55.

Bohn, W., Rulter, G., Hohenberg, H., Mannweiler, K. and Nobis, P. (1986): Involvement of actin filaments in the budding of measles virus: studies on cytoskeletons of infected cells. *Virol.* **149**, 91-106.

Bouteille, M., Fountaine, C., Vedrenne, C. and Delanie, J. (1965): Sur un cas d'encephalite subaigue a inclusions. Etude anatomoclinique et ultrastructurale. *Revve Neruol.* **113**, 454-458.

Bowen, H.A. and Lyles, D.S. (1982): Kinetics of incorporation of Sendai virus proteins into host plasma membranes and virions. *Virol.* **121**, 1-11.

Brodsky, A.L. (1972): Atypical measles: severe illness in recipients of killed measles virus vaccine upon exposure to natural infection. *J. Am. Med. Assoc.* **222**, 1415-1416.

Buckland, R., Gerald, C., Barker, R. and Wild, T.F. (1987): Fusion glycoprotein of measles virus: nucleotide sequence of the gene and comparison with other paramyxoviruses. *J. Gen. Virol.* **68**, 1695-1703.

Buckland, R., Gerald, C., Barker, D. and Wild, T.F. (1988): Cloning and sequence of the nucleoprotein gene of measles virus (Halle) strain. *Nucleic Acids Research.* **16**, 11821-11825.

Buckland, R., Giraudon, P. and Wild, T.F. (1989): Expression of measles virus nucleocapsid protein in *Escherichia coli*: use of deletion mutants to locate the antigenic sites. *J. Gen. Virol.* **70**, 435-441.

Buckland R. and Wild, T.F. (1989): Leucine zipper motif extends. *Nature*. **338**, 547.

Buckland, R., Malvoisin, E., Baeuverger, P. and Wild, T.F. (1992): A leucine zipper structure present in the measles virus fusion protein is not required for its tetramerization but is essential for fusion. *J. Gen. Virol.* **73**, 1703-1707.

Buchholz, C. J., Schneider, U., Devaux, P., Gerlier, D. and Cattaneo, R. (1996): Cell entry by measles virus: long hybrid receptors uncouple binding from membrane fusion. *J. Virol.* **70**, 3716-3723.

Burnstein, T., Jacobson, L.B., Zeman, W. and Chen, T.T. (1974): Persistent infection of BS-C-1 cells by defective measles virus derived from SSPE. *Infect. Immunol.* **10**, 1378-1382.

Cane, P.A., Matthews, D.A. and Pringle, C.R. (1993): Frequent polymerase errors observed in a restricted area of clones derived from the attachment (G) protein gene of respiratory syncytial virus. *J. Virol.* **67**, 1090-1093.

Carlsen, S.R., Peluso R.W. and Moyer, S.G. (1985): *In-vitro* replication of Sendai virus wild-type and defective interfering particle genome RNA's. *J. Virol.* **54**, 493-500.

Carrigan, D.R. (1985): Round cell variant of measles virus: spontaneous conversion from productive to cell-associated state of infection. *Virol.* **144**, 337-350.

Carrigan, D.R. (1986): Round cell variant of measles virus: neurovirulence and pathogenicity of acute encephalitis in newborn hamsters. *Virol.* **148**, 349-359.

Carter, C.H., Conway, T.J. *et al.* (1962): Serologic response of children to inactivated measles virus. *J. Am. Med. Assoc.* **179**, 848-853.

Carter, C.H., Black, F.L. and Schluederburg, A. (1973): Nucleus associated RNA in measles virus infected cells. *Biochem. Biophys. Res. Commun.* **54**, 411-416.

Carter, M.J., Willcocks, M.M. and ter Meulen, V. (1983): Defective translation of measles virus matrix protein in an SSPE cell line. *Nature*. **305**, 153-155.

Carter, M.J. and Milton, I.D. (1993): An inexpensive and simple method for DNA purification on silica particles. *Nucleic Acid Research.* **21**, 1044.

Casteneda, S.J. and Wong, T.C. (1990): Leader sequence distinguishes between translatable and encapsidated measles virus RNAs. *J. Virol.* **64**, 222-230.

Cattaneo, R., Schmid, A., Rebmann, G., Baczko, K., ter Meulen, V., Bellini, W.J., Rozenblatt, S. and Billeter, M.A. (1986): Accumulated measles virus mutations in a case of SSPE: interrupted matrix protein reading frame and transcription alteration. *Virol.* **154**, 97-107.

Cattaneo, R., Rebmann, G., Baczko, K., ter Meulen, V. and Billeter, M.A. (1987): Altered ratio of measles virus transcripts in diseased human brains. *Virol.* **160**, 523-526.

Cattaneo, R., Schmid, A., Billeter, M.A., Sheppard, R.D. and Uedem, S.A. (1988a): Multiple viral mutations rather than host factors cause defective measles virus gene expression in an SSPE cell line. *J. Virol.* **62**, 1388-1397.

Cattaneo, R., Schmid, A., Eschle, D., Baczko, K., ter Meulen, V. and Billeter, M.A. (1988b): Biased hypermutation and other genetic changes in defective measles virus in human brain infections. *Cell.* **55**, 255-265.

Cattaneo, R., Kaelin, K., Baczko, K. and Billeter, M.A. (1989a): Measles virus editing provides an additional cysteine-rich protein. *Cell.* **56**, 759-764.

Cattaneo, R., Schmid, A. Spielhofer, P., Kaelin, K., Baczko, K., ter Meulen, V., Pardowitz, J., Flanagan, S., Rima, B.K., Yolem, S.A. and Billeter, M.A. (1989b): Mutated and hypermutated genes of persistent measles virus which cause lethal human brain diseases. *Virol.* **173**, 415-425.

Cattaneo, R. and Rose, J.K. (1993): Cell fusion by the envelope glycoproteins of persistent measles virus which cause lethal human brain disease. *J. Virol.* **67**, 1493-1502.

Chambers, P., Pringle, C.R. and Easton, A.J. (1992): Sequence analysis of the gene encoding the fusion glycoprotein of pneumovirus of mice suggests possible conserved secondary structure elements in paramyxovirus fusion glycoproteins. *J. Gen. Virol.* **73**, 1717-1724.

Chattopadyay, D. and Banerjee, A.K. (1987): Two separate domains within vesicular stomatitis virus phosphoprotein support transcription when added in trans. *Proc. Natl. Acad. Sci. USA.* **84**, 8932-8936.

Chattopadyay, D and Banerjee, A.K. (1988): NH_2-terminal acid region of the phosphoprotein of VSV can be functionally replaced by tubulin. *Proc. Natl. Acad. Sci. USA.* **85**, 7977-7981.

Chen, R.T., Markowitz, L.E., Albrecht, P., Stewart, J.A., Mofenson, L.M., Preblud, S.R. and Orenstein, W.A.(1990): Measles antibodies: re-evaluation of protective titres. *J. Infect. Dis.* **162**, 1036-1042.

Cherry, J.D., Feigin, R.D. *et al.*. (1972): Atypical measles in children previously immunised with attenuated measles virus vaccines. *Paediatrics*. **50**, 712-717.

Cherry, J.D. (1992): Measles. In: Feigin, R.D., Cherry, J.D. (Eds). *Paediatric infectious diseases*. **3rd Edition**, 1595.

Choppin, P.W. and Compans, R. (1975): Reproduction of paramyxoviruses. In: Fraenkal-Conrat, H. and Wagner, R.R. (Eds). *Comprehensive Virology*. **4**, 95-178. Plennum Press, New York.

Choppin, P.W. and Scheid, A. (1980): The role of viral glycoproteins in adsorption, penetration and pathogenicity of viruses. *Rev. Infect. Dis.* **2**, 40-61.

Clements, C.J. and Cutts, F.T. (1995): The epidemiology of measles: thirty years of vaccination. In: ter Meulen, V. and Billeter, M.A. (Ed) Measles Virus. *Current topics in microbiology and immunology.* **191**, 13-33.

Collins, P. (1991): The molecular biology of human respiratory syncytial virus of the genus pneumovirus. In: Kingsbury, D.W. (Ed). *The paramyxoviruses*. Plennum Press, New York. 103-162.

Compans, R.W., Holmes, K.V., Dales, S. and Choppin, P.W. (1966): An electron microscopic study of moderate and virulent virus-cell interactions of the parainfluenza virus SV5. *Virol.* **30**, 411-426.

Connolly, J.H., Allen, I.V., Hurwitz, L.J. and Miller, J.H.D. (1967): Measles virus antibodies and antigens in SSPE. *Lancet I.* 542-544.

Cosby, S.L., Macquaid, S., Taylor, M.J., Bailey, M., Rima, B.K., Martin, S.J. and Allen, I.V. (1989): Examination of eight cases of multiple sclerosis and 56 neurological and non-neurological controls for genomic sequences of measles virus. *J. Gen. Virol.* **70**, 2027-2036.

Cosby, S.L. (1993): Measles virus: a possible role in various diseases of unknown origin. *Microbiol. Eur.* **5**, 22-26.

Crowley, J.C., Dowling, P., Menonna, J., Silverman, J.I., Schuback, D., Cook, S.D. and Blumberg, B.M. (1988): Sequence variability and function of measles virus 3' and 5' end and intercistronic regions. *Virol.* **164**, 498-506.

Curran, J., Boeck, R. and Kolakofsky, D. (1991): The Sendai virus P gene expresses both an essential protein and an inhibitor of RNA synthesis by shuffling modules via messenger RNA editing. *EMBO.* **10**, 3079-3085.

Das, T., Schuster, A., Schneider-Schaulies, S. and Banerjee, A.K. (1995): Involvement of cellular kinase II in the phosphorylation of measles virus phosphoprotein: identification of phosphorylation sites. *Virol.* **211**, 218-226.

Dayan, A.D., Gostling, J.V.T., Greaves, J.L., Stevens, D.W. and Woodhouse, M.A. (1967): Evidence of a pseudomyxovirus in the brain in sub-acute sclerosing leucoencephalitis. *Lancet.* 980-981.

Dingwall C. and Laskey, R.A. (1991): Nuclear targeting sequences - a consensus? *Trends Biochem. Sci.* **16**, 478-481.

Dorig, R.E., Marcil, A., Chopra, A. and Richardson, C. D. (1993): The human CD46 molecule is a receptor for measles virus (Edmonston strain). *Cell.* **75**, 295-305;

Dubois-Dalcq, M., Barbosa, L.H., Hamilton, R. and Sever, J.L. (1974a): Comparison between productive and latent subacute sclerosing panencephalitis virus infection *in-vitro*. *Lab. Invest.* **30**, 241-250.

Dubois-Dalcq, M., Coblentz, J.M. and Pleet, A.B. (1974b): Subacute sclerosing panencephalitis: unusual nuclear inclusions and lengthy clinical course. *Arch. Neurol.* **31**, 355-364.

Dunster, L.M., Schneider-Schaulies, J., Loffler, S., Lankes, W., Schwartz-Albiez, R., Lottspeich, F. and ter Meulen, V. (1994): Moesin: a cell membrane protein linked with susceptibility to measles virus infection. *Virol.* **198**, 265-274.

Dunster, L.M., Schneider-Schaulies, J., Dehoff, M.H., Holers, V.M., Schwarz-Albeiz, R. and ter Meulen, V. (1995) Moesin, and not the functional homologue (Crry/p65) of human membrane cofactor protein (CD46) is involved in the entry of measles virus (Edmonston strain) into susceptible cell lines. *J. Gen. Virol.* **76**, 2085-2089.

Ecker, A., ter Meulen, V., Baczko, K. and Schneider-Schaulies, S. (1995): Measles virus-specific dsRNA's are targets for unwinding/modifying activity in neural cells *in-vitro*. *J. Neuroviol.* **1**, 92-100.

Edmonston, M. B. (1990): Mild measles and secondary vaccine failure during a sustained outbreak in a highly vaccinated population. *J. Am. Med. Assoc.* **263**, 2467-2471.

Ehrnst, A.C. (1975): Characterisation of measles virus-specific cytotoxic antibodies by use of a chronically infected cell line. *J. Immunol.* **114**, 1077-1082.

Ehrnst, A.C. (1978): Separate pathways of C activation by measles virus cytotoxic antibodies: subclass analysis and capacity of F (ab) molecules to activate C via the alternative pathway. *J. Immunol.* **121**, 1206-1212.

Enami, M., Sata, T.A. and Sugiura, A. (1989): Matrix protein of cell-associated sclerosing panencephalitis viruses. *J. Gen. Virol.* **70**, 2191-2196.

Enders, J.F. and Peebles, T.C. (1954): Propagation in tissue cultures of cytopathogenic agents from patients with measles. *Proc. Soc. Exp. Bio. Med.* **86**, 277-286.

Fooks, A.R., Stephenson, J.R., Warnes, A., Dowsett, A.B., Rima, B.K. and Wilkinson, G.W.G. (1993): Measles virus nucleocapsid protein expressed in insect cells assembles into nucleocapsid-like structures. *J. Gen. Virol.* **74**, 1439-1444.

Fooks, A.R., Schadeck, E., Liebert, U.G., Dowsett, A.B., Rima, B.K., Steward, M., Stephenson, J.R. and Wilkinson, G.W.G. (1995): High level expression of measles virus nucleocapsid protein by using a replication-deficient adenovirus vector: Induction of an MHC-1 restricted CTL response and protection in a murine model. *Virol.* **210**, 456-465.

Follett, E.A.C., Pringle, C.R. and Pennington, T.H. (1976): Events following the infection of enucleated cells with measles virus. *J. Gen. Virol.* **32**, 163-175.

Freeman, J.M., Magoffin, R.L., Lennette, E.H. and Herdon, R.M. (1967): Additional evidence of the relationship between SSPE and measles virus. *Lancet ii.* 129-131.

Freeman, R.B. (1984): Native disulphide bond formation in protein biosynthesis: evidence for the role of protein disulphide isomerase. *Trends Biochem. Sci.* **9**, 438-441.

Fujinami, R.A. and Oldstone, B.A. (1979): Antiviral antibodies reacting on the plasma membrane alter measles virus expression inside the cell. *Nature*. **279**, 529-530.

Garcia-Barreno, B., Portela, A., Delgado, T., Lopez, J.A. and Melero, J.A. (1990): Frame shift mutations as a novel mechanism for the generation of neutralization resistant mutants of human respiratory syncytial virus. *The EMBO J.* **9**, 4181-4187.

Garenne, M., Leroy, O., Beau, J.P. and Sene, I. (1991): Child mortality after high-titre measles vaccines: prospective study in Senegal. *Lancet*. **338**, 903-907.

Gerald, C., Buckland, R., Barker, R., Freeman, G. and Wild, T.F. (1986): Measles virus haemagglutinin gene: cloning, complete nucleotide sequence analysis and expression in COS cells. *J. Gen. Virol*. **67**, 2695-2703.

Gething, M.J., White, J.M. and Waterfield, M.D. (1978): Purification of the fusion protein of Sendai virus: analysis of the NH_2- terminal sequence generated during precursor activation. *Proc. Natl. Acad. Sci. USA*. **75**, 2737-2740.

Giraudon, P., Jacquier, M.F. and Wild, T.F. (1988): Antigenic analysis of African measles field isolates: identification and localisation of one conserved and two variable epitope sites on the NP protein. *Virus Res.* 137-153.

Gombart, A.F., Hirano, A. and Wong, T.C. (1993): Conformational maturation of measles virus nucleocapsid protein. *J. Virol.* **67**, 4133-4141.

Graves, M.C., Silver, S.H. and Choppin, P.W. (1978): Measles virus polypeptide synthesis in infected cells. *Virol.* **86**, 254-263.

Greer, P.A., Hasel, K.W. and Millward, S. (1986): Cloning and *in-vitro* expression of the measles virus matrix gene. *Biochem. Cell. Biol.* **65**, 1038-1043.

Griffin, D.E., Cooper, S.J., Hirsch, R.L., Johnson, R.T., Lindo de Soriano, I., Roedenbeck, S. and Vaisberg, A. (1985): Changes in plasma IgE levels during complicated and uncomplicated measles virus infections. *J. Allergy Clin. Immunol.* **76**, 206-213.

Griffin, D.E., Ward, B.J., Jauregui, E., Johnson, R.T. and Vaisberg, A. (1989): Immune activation during measles. *N. Engl. J. Med.* **320**,1667-1672.

Griffin, D.E., Ward, B.J., Jauregui, E., Johnson, R.T. and Vaisberg, A. (1990a): Natural killer cell activity during measles. *Clin. Exp. Immunol.* **81**, 218-224.

Griffin, D.E., Ward, B.J., Jauregui, E., Johnson, R.T. and Vaisberg, A. (1990b): Immune activation during measles: IFN-γ and neopterin in plasma and cerebrospinal fluid in complicated and uncomplicated disease. *J. Infect. Dis.* **161**, 449-453.

Griffin, D.E., Ward, B.J., Juaregui, E., Johnson, R.T. and Vaisberg, A. (1992): Immune activation during measles: β_2-microglobulin in plasma and cerebrospinal fluid in complicated and uncomplicated disease. *J. Infect. Dis.* **166**, 1170-1173.

Griffin, D.E. and Ward, B.J. (1993): Differential CD4 T-cell activation in measles. *J. Infect. Dis.* **168**, 275-281.

Hajjar, A.M. and Linial, M.L. (1995): Modification of retroviral RNA by double-stranded RNA adenosine deaminase. *J. Virol.* **69**, 5878-5882.

Hall, W.W., Lamb, R.A. and Choppin, P.W. (1979): Measles and subacute sclerosing panencephalitis virus proteins: lack of antibody to the M protein in patients with SSPE. *Proc. Natl. Acad. Sci. USA.* **76**, 2047-2051.

Halsey, N.A., Boulos, R., Mode F., Andre, J., Bowman, L., Yaeger, R.G., Toureau. S., Rohde, J. and Boulos, C. (1985): Response to measles vaccine in Haitian infants 6-12 months old. *N. Engl. J. Med.* **313**, 544-549.

Hamaguchi, M., Nishikawa, K., Toyoda, T., Yoshida, T., Hanaichi, T. and Nagai, Y. (1985): Transcriptive complex of NDV II: Structural and functional assembly associated with the cytoskeletal framework. *Virol.* **147**, 295-308.

Harty, R.N. and Palese, P. (1995): Measles virus phosphoprotein (P) required the NH_2- and COOH-terminal domains for interactions with the nucleoprotein (N), but only the COOH-terminus for interactions with itself. *J. Gen. Virol.* **76**, 2863-2867.

Heggeness, M.H., Scheid, A. and Choppin, P.W. (1981): The relationship of conformational changes in the Sendai virus nucleocapsid to proteolytic cleavage of the nucleocapsid polypeptide. *Virol.* **114**, 555-562.

Henderson, G.W., Laird, C., Dermott, E. and Rima, B.K. (1995): Characterisation of Mapuera virus: structure, proteins and nucleotide sequence of the gene encoding the nucleocapsid. *J. Gen. Virol.* **76**, 2509-2518.

Hirano, A. (1992a): Subacute sclerosing panencephalitis virus dominantly interferes with replication of wild-type measles virus in a mixed infection: implication for viral persistence. *J. Virol.* **66**, 1891-1898.

Hirano, A., Wang, A.H., Gombart, A.F. and Wong, T.C. (1992b): The matrix proteins of neurovirulent SSPE virus and its acute measles virus progenitor are functionally different. *Proc. Natl. Acad. Sci. USA.* **89**, 8745-8749.

Hirano, A. Ayata, M, Wong, A.H. and Wong, T.C. (1993): Functional analysis of matrix proteins expressed from the cloned genes of measles virus variants that cause SSPE reveal common defects in nucleocapsid binding. *J. Virol.* **67**, 1848-1853.

Homma, M., Tashiro, M., Konno, H., Ohara, Y., Hino, M. and Takase, S. (1982): Isolation and characterisation of SSPE virus (Yamagata-1 strain) from a brain autopsy. *Mircrobio. Immunol.* **26**, 1195-1202.

Hope-Simpson, R.E. (1952): Infectiousness of communicable diseases in the household (measles, chicken pox and mumps). *Lancet ii.* 549-554.

Horikami, S.M. and Moyer, S.A. (1991): Synthesis of leader RNA and editing phosphoprotein mRNA during transcription by purified measles virus. *J. Virol.* **65**, 5342-5347.

Horta-Barbosa, L., Hamilton, R., Witting, B., Fucillo, D.A., Sever, J.I. and Vernon, M.L. (1971): SSPE: isolation of suppressed measles virus from lymph node biopsy. *Science.* **173**, 840-841.

Hu, A. and Norrby, E. (1994a): Role of individual cysteine residues in the processing and antigenicity of the measles virus haemagglutinin protein. *J. Gen. Virol.* **75**, 2173-2181.

Hu, A., Cattaneo, R., Schwartz, S. and Norrby, E. (1994b): Role of N-linked oligosaccharide chains in the processing and antigenicity of measles virus haemagglutinin protein. *J. Gen. Virol.* **75**, 1043-1052.

Hu, A., Kovamees, J. and Norrby, E. (1994c): Intracellular processing and antigenic maturation of measles virus haemagglutinin protein. *Arch. Virol.* **136**, 239-253.

Hu, A., Cathomen, T., Cattaneo, R. and Norrby, E. (1995): Influence of N-linked oligosaccharide chains on the processing, cell surface expression and function of the measles virus fusion protein. *J. Gen. Virol.* **76**, 705-710.

Huang, A.S. and Baltimore, D. (1970): Defective viral particles and viral disease processes. *Nature.* **226**, 325-327.

Huber, M., Cattaneo, R., Speilhofer, P., Orvell, C., Norrby, E., Messerli, M., Perriard, J.C. and Billeter, M.A. (1991): Measles virus phosphoprotein retains the nucleocapsid protein in the cytoplasm. *Virol.* **185,** 299-308.

Hughes, I., Jenney, M.E., Newton, R.W. Morris, D.J. and Klapper, P.E. (1993): Measles encephalitis during immunosuppression therapy for acute lymphoblastic leukaemia. *Arch. Dis. Child.* **68**, 775-778.

Hull, J.D., Krah, D.L. and Choppin, P.W. (1987): Resistance of a measles virus mutant to fusion inhibition oligopeptides is not associated with mutations in the fusion peptide. *Virol.* **159**, 368-372.

Hummel, K.B., Vanchiere, J.A. and Bellini, W.J. (1994): Restriction of the fusion protein mRNA as a mechanism of measles virus persistence. *Virol.* **202**, 665-672.

Ilonen, J., Makela, M.J. and Salmi, B. (1990): Cloning of human T-cells specific for measles virus haemagglutinin and nucleocapsid proteins. *Clin. Exp. Immunol.* **81**, 212-217.

Jacobson, S., Sekaly, R.P., Jacobson, C.L., McFarland, H.F. and Long, E.O. (1989): Class II-restricted presentation of cytoplasmic measles virus antigens to cytotoxic T cells. *J. Virol.* **63**, 1756-1762.

Jacobson, S., Richet, J.R., Biddison, W.E., Satinsky, A., Hartzman, R.J. and McFarland, H.F. (1984): Measles virus-specific CD4+ human cytotoxic T cell clones are restricted by class II human lymphocyte antigens. *J. Immunol.* **133**, 754-757.

Katz, M, Oyanagi, S. and Koproswski, H. (1969): SSPE: structures resembling myxovirus nucleocapsids in cells cultured from brain. *Nature*. **222**, 888-899.

Katz, M. (1995): Clinical spectrum of measles. In: ter Meulen, V. and Billeter, M.A. (Eds) Measles Virus. *Current Topics in Microbiology and Immunology*. **191**, 1-12.

Khatchikian, D., Orlich, M. and Rott, R. (1989): Increased viral pathogeneticity after insertion of a 28S ribosomal RNA sequence into the haemagglutinin gene of an influenza virus. *Nature*. **340**, 156-157.

Kim, S.H., Suddath, F.L., Quigley, G.J., McPherson, A., Sussman, J.L., Wang, A.H.J., Seeman, N.C. and Rich, A. (1974): Three-dimentional tertiary structure of yeast phenlyalanine transfer RNA. *Science*. **185**, 435-440.

King, J.C., Lichnestein, R., Feigelman, P., Luna, C., Permutt, J.C. and Patel, J. (1993): Measles, mumps and rubella antibodies in vaccinated Baltimore children. *Amer. J. Dis. Child.* **147**, 558-560.

Kingsbury, D.W., Bratt, M.A., Choppin, P.W., Hanson, R.P., Hosaka, Y., Meulen, T., Norrby, E., Plowright, W., Rott, R. and Wunner, W. H. (1978): Paramyxoviridae. *Intervirology*. **10**, 137-152.

Koetzner, C.A., Parker, M.M., Sturman, R.L. and Masters, P.S. (1992): Repair and mutagenesis of the genome of a deletion mutant of the coronavirus mouse hepatitis virus by targete RNA recombination. *J. Virol.* **66**, 1841-1848.

Kolakofsky, D. and Blumberg, B.M. (1982): A model fro the control of non-segmented negative-strand virus genome replication. In: Mahy, B.W.J., Minson, A.C. and Darby, G.K.(Eds). *Viral persistence symposium*. **33**. Cambridge University Press, Cambridge. 203-212.

Kolakofsky, D., Vidal, S. and Curran, J. (1991): Paramyxovirus RNA synthesis and phosphoprotein gene expression. In: Kingsbury, D.W. (Ed). *The paramyxoviruses*. Plennum Press, New York. 215-233.

Komase, K., Rima, B.K., Pardowitz, I., Kunz, C., Billeter, M.A., ter Meulen, V. and Baczko, K. (1995): A comparison of nucleotide sequence of measles virus L genes derived from wild-type viruses and SSPE brain tissue. *Virol.* **208**, 795-799.

Kovamees, J., Blixenkrone-Moller, M., Sharma, B., Orvel, C. and Norrby, E. (1991a): The nucleotide sequence and predicted amino acid sequence of the attachment protein of canine distemper virus. *Virus Res.* **19**, 223-231.

Kovamees, J., Blixenkrone-Moller, M., Sharma, B., Orvel, C. and Norrby, E. (1991b): The nucleotide sequence deduced amino acid composition of the haemagglutinin and fusion proteins of the morbillivirus phocid distemper virus. *J. Gen. Virol.* **72**, 2959-2966.

Lairmore, M.D., DiGeorge, A.M., Conrad, S.F., Trevino, A.V., Lal, R.B. and Kaumaya, P.T.P. (1995): Human T-lymphotropic virus type I peptides in chimaeric and multivalent constructs with promiscuous T-cell epitopes enhances immunogenically and overcomes genetic restriction. *J. Virol.* **69**, 6077-6080.

Lamb, R.A. and Choppin, P.W. (1977): The synthesis of Sendai virus polypeptides in infected cells II: intracellular distribution of polypeptides. *Virol.* **81**, 371-381.

Lecouturier, V., Fayolle, J., Caballero, M., Carabana, J., Celma, M.L., Fernandez-munoz, R., Wild, T.F. and Buckland, R. (1996): Indentification of two amino acids in the haemagglutinin glycoprotein of measles virus (MV) that govern heamadsorption, HeLa cell fusion, and CD46 downregulation: phenotypic markers that differentiate vaccine and wild-type MV strains. *J. Virol.* **70**, 4200-4204.

Leopardi, R., Hukkanen, V., Vainionpaa, R. and Salmi, A.A. (1993): Cell proteins bind to sites within the 3' non-coding region and the positive-strand leader sequence of measles virus RNA. *J. Virol.* **67**, 785-790.

Li, J.K.K., Mryakawa, T. and Fox, C.F. (1980): Protein organisation in newcastle disease virus as revealed by perturbant treatment. *J. Virol.* **34**, 268-271.

Liebert, U.G., Flanagan, S.G., Loffler, S., Baczko, K., ter Meulen, V. and Rima, B.K. (1994): Antigenic determinants of the measles virus haemagglutinin associated with neurovirulence. *J. Virol.* **68**, 1486-1493.

Ling, R. and Pringle, C.R. (1989): Polypeptides of pneumovirus of mice II: characterisation of the glycoproteins. *J. Gen. Virol.* **70**, 1441-1452.

Liston, P and Briedis, D.J. (1994): Measles virus V protein binds zinc. *Virol.* **198**, 399-404.

Liston, P and Briedis, D.J. (1995): Ribosomal frameshifting during translation of measles virus phosphoprotein mRNA is capable of directing synthesis of a unique protein. *J. Virol.* **69**, 6742-6750.

Liszewski. M.K., Post, T.W. and Atkinson, J.P. (1991): Membrane cofactor protein (MCP or CD46): newest member of the regulators of complement activation gene cluster. *Annu. Rev. Immunol.* **9**, 431-455.

Lucas, C.J., Biddison, W.E., Nelson, D.L. and Shaw, S. (1982): Killing of measles virus infected cells by human cytotoxic T-cells. *Infect. Immunol.* **38**, 226-239.

Maisner, A., Schneider-Schulies, J., Lisezwski, M.K., Atkinson, J.P. and Herrler, G. (1994): Binding of measles virus to membrane cofactor protein (CD46): importance of disulphide bonds and N-glycans for the receptor function. *J. Virol.* **68**, 6299-6304.

Maisner, A. and Herrler, G. (1995): Membrane cofactor protein with different types of N-glycans can serve as measles virus receptor. *Virol.* **210**, 479-481.

Malvoisin, E. and Wild, T.F. (1993): Measles virus glycoproteins: studies on the structure and interaction of the haemagglutinin and fusion proteins. *J. Gen . Virol.* **74**, 2365-2372.

Malvoisin, E. and Wild, T. F. (1994): The role of N-glycosylation in cell fusion induced by a vaccinia recombinant virus expressing both measles virus glycoproteins. *Virol.* **200**, 11-20.

Manchester, M., Liszewski, M.K., Atkinson, J.P. and Oldstone, M.B.A. (1994): Multiple isoforms of CD46 (membrane cofactor protein) serves as receptors for measles virus. *Proc. Natl. Acad. Sci. USA*, **91**, 2161-2165.

Manchester, M., Valsimakis, A., Kaufman, R., Liszewski, M.K., Alvarez, J., Atkinson, J.P., Lubin, D.M. and Oldstone, M.B.A. (1995): Measles virus and C3 binding sites are distinct on membrane cofactor protein. *Proc. Natl. Acad. Sci. USA*. **92**, 2303-2307.

Markowitz, L.E., Preblud, S.R., Fine P.E., Orenstein, W.A. (1990): Duration of live measles vaccine induced immunity. *Paediatr. Infect. Dis. J.* **9**, 101-110.

Markwell, M.A.K. and Fox, C.F. (1980): Protein-protein interaction with paramyxoviruses identified by native disulphide bonding or reversible chemical cross-linking. *J. Virol.* **33**, 152-166.

Matthews, R.E.F. (1982): Classification and nomenclature of viruses. *Intervirology*. **17**, 104-105.

Mathiesen T., Hammarstrom, L., Fridell., Linde, A., Wirsen, G., Smith, C.I., Norrby, E. and Wahren, B. (1990): Aberrant IgG subclass distribution to measles in healthy seropositive individuals, in patients with SSPE and in immunoglobulin-deficient patients. *Clin. Exp. Immunol.* **80**, 202-205.

Matlin, K. and Simons, K. (1984): Sorting of an apical plasma membrane glycoprotein occurs before it reaches the cell surface in cultured epithelial cells. *J. Cell. Biol.* **99**, 2131-2139.

Meissner, N.N. and Koschel, K. (1995): Down-regulation of endothelin receptor mRNA synthesis in C6 rat astrocytoma cells by persistent measles virus and canine distemper virus infections. *J. Virol.* **69**, 5191-5194.

Meyers, G., Tautz, N., Dubovi, E.J. and Thiel, H.J. (1991): Viral cytopathogenesis correlated with integration of ubiquitin coding sequences. *Virol.* **180**, 602-616.

Miller, C.A. and Carrigan, D.R. (1982): Reversible repression and activation of measles virus infection in neural cells. *Proc. Natl. Acad. Sci. USA.* **79**, 1629-1633.

Misek, D.E., Bard, E. and Rodriguez-Boulan, E. (1984): biogenesis of epithelial cell polarity: intracellular sorting and vectorial exocytosis of an apical plasma membrane glycoprotein. *Cell.* **39**, 537-546.

Modlin, J.F., Halsey, N.A., Eddin, D.L., Conrad, J.L., Jabbour, J.T., Chien, L. and Robinson, H. (1979): Epidemiology of SSPE. *J. Paediatr.* **9**, 231-236.

Monroe, F.F. and Schlefinger, F. (1983): RNA's from two independently isolate defective interfering particles of Sindbis virus containing cellular tRNA sequence at their 5' end. *PNAS USA.* **80**, 3279-3283.

Morgan, E.M., Re, G.G. and Kingsbury, D.W. (1984): Complete sequence of the Sendai virus nucleocapsid protein gene from a cloned insert. *Virol.* **135**, 279-287.

Morgan, E.M. (1991): Evolutionary relationships of paramyxovirus nucleocapsid-associated proteins. In: Kingsbury, D.W. (Ed). *The paramyxoviruses*. Plennum Press, New York. 163-176.

Mori, T., Sasaki, H., Hashimoto, H. and Makino, S. (1993): Molecular cloning and complete nucleotide sequence of genomic RNA of the AIK-C strain measles virus. *Virus Genes*. **7**, 67-81.

Morrison, T. and Portner, A. (1991): Structure, function and intracellular processing of the glycoproteins in the family paramyxoviridae. In: Kingsbury, D.W. (Ed). *The paramyxoviruses*. Plennum Press, New York. 347-382.

Moyer, S.A., Baker, S.C. and Lessard, J.L. (1986): Tubulin: a factor necessary for the synthesis of both Sendai and vesicular stomatitis virus RNAs. *Proc. Natl. Acad. Sci. USA.* **83**. 5405-5409.

Moyer, S.A., Baker, S.C. and Horikama, S.M. (1990): Host cell proteins required for measles virus reproduction. *J. Gen. Virol.* **71**, 775-783.

Moyer, S.A. and Horikami, S.M. (1991): The role of viral and host cell proteins in paramyxovirus transcription and replication. In: Kingsbury, D.W. (Ed) *The paramyxoviruses.* Plennum press, New York. 249-274.

Murphy, F.A., Fauquet, C.M., Bishop, D.H.L., Ghabrial, S.A., Jarvis, A.W., Martelli, G.P., Mayo, M.A. and Summers, M.D. (1995): Virus Taxonomy: classification and nomenclature of viruses. 6th Report of the International committee on the Taxonomy of Viruses. *Arch. Virol.* **supp. 10**,268-274.

Naniche, D., Wild, T.F., Raboudin-Combe, C. and Gerlier, D. (1992): A monoclonal antibody recognises a human cell surface glycoprotein involved in measles virus binding. *J. Gen. Virol.* **73**, 2617-2624.

Naniche, D., Variro-Krishnan, G., Cervoni, F., Wild, T.F., Rossi, B., Rabourdin-Combe, C. and Gerlier, D. (1993a): Human membrane cofactor protein (CD46) acts as a cellular receptor for measles virus. *J. Virol.* **67**, 6025-6032.

Naniche, D., Wild, T.F., Rabourdin-Combe, C. and Gerlier, D. (1993b): Measles virus haemagglutinin induced down-regulation of gp 57/67 molecule involved in virus binding. *J. Gen. Virol.* **74**,1073-1079.

Nishikura, K. (1992): Modulation of double-stranded RNA's *in-vivo* by RNA duplex unwindase. *Ann. N.Y. Acad Sci.* **660,** 240-250.

Norrby, E., Enders-Ruckle, G. and ter Meulen, V. (1975): Differences in the appearance of antibody to structural components of measles virus after immunisation with inactivated and live virus. *J. Infect. Dis.* **132**, 262-269.

Novick, N. and Hoekstra, D. (1988): Membrane penetration of Sendai virus glycoprotein during the early stages of fusion with liposomes as determinants by hydrophobic photoaffinity labelling. *Proc. Acad. Natl. Sci. USA.* **85**, 7433-7437.

Oddo, F.G., Flaccomio, R. and Sinatra, A. (1961): 'Giant cell' and 'strand-forming' cytopathic effects of measles virus lines by serial propagation with diluted or concentrated inoculum. *Virol.* **13**, 550-553.

O'Hara, P.J., Nichol, S.T., Horodyski, A. and Holland, J.J. (1984): Vesicular stomatitis virus defective interfering paticles can contain extensive genomic sequence rearrangements and base substitution. *Cell.* **36**, 915-924.

Outlaw, M. and Pringle, C.R. (1995): Sequence variation within an outbreak of measles virus in the Coventry area during spring/summer 1993. *Virus Res.* **39**, 3-11.

Patterson, J.B. and Samual, C.E. (1995): Expression and regulation by interferon of a double-stranded RNA-specific adenosine deaminase from human cells: evidence for two forms of the deaminase. *Mol. Cel. Biol.* **15**, 5376-5388.

Pedersen, I.R., Mordhorst, C.H., Glikmann, G. and von Magnus, H. (1989): Subclinical measles infection in vaccinated sero-positive individuals in Arctic Greenland. *Vaccine*. **7**, 345-348.

Poch, O., Blumberg, B.M., Bougeleret, L. and Tordo, N. (1990): Sequence comparison of five polymerase (L protein) of unsegmented negative strand RNA viruses: theoretical assignment of functional domains. *J. Gen. Virol.* **71**, 1153-1162.

Polson, A.G., Bass, B.L. and Casey, J.L. (1996): RNA editing of hepatitis delta virus antigenome of dsRNA adenosine deaminase. *Nature*. **380**, 454-456.

Pfeiffer, S., Fuller, S.D. and Simons, K. (1985): Intracellular sorting and basolateral appearance of the G protein of VSV in MDCK cells. *J. Cell. Biol.* **101**, 470-476.

Pringle, C.R. (1991): The genetics of Paramyxoviruses. In: Kingsbury, D.W. (Ed). *The paramyxoviruses*. Plennum Press, New York, 1-39.

Pringle, C.P. (1995): In: Gibbs, A., Calisher, C.H. and Garcia-Arenal, F. (Eds) *Molecular basis of virus evolution.* Cambridge University Press.

Radecke, F., Spielhofer, P., Schneider, H., Kaelin, K., Huber, M., Dotsch, C., Christiansen, G. and Billeter, M.A. (1995): Rescue of measles virus from cloned DNA. *The EMBO J.* **14**, 5773-5784.

Rammohan, K.W., McFarland, H.F., Bellini, W.J., Ghenens, J. and McFarlin. (1983): Antibody-mediated modification of encephalitis induced by hamster neurotropic measles virus. *J. Infect. Dis.* **147**, 546-550.

Rataul, S.M., Hirano, A. and Wong, T.C. (1992): Irreversible modification of measles virus RNA *in-vitro* by nuclear RNA-unwinding activity in human neuroblastoma cells. *J. Virol.* **66**, 1769-1773.

Richardson, C., Scheid, A. and Choppin, P. (1980): Specific inhibition of paramyxovirus replication by oligopeptides with amino acid sequence similar to those at N-termini of the F1 or HA2 viral polypeptides. *Virol.* **105**, 205-222.

Richardson, C., Hull, D., Greer, P., Hassle, K., Berkovich, A., Englund, G., Bellini, W.J., Rima. B.K. and Lazzarini, R. (1986): The nucleotide sequence of the mRNA encoding the fusion protein of measles virus (Edmonston strain): a comparison of fusion proteins from several different paramyxoviruses. *Virol.* **155**, 508-523.

Riley, E.C., Murphy, G. and Riley, R.L. (1978): Airborne spread of measles in a suburban elementary school. *Am. J. Epidemiol.* **107**, 421-432.

Rima, B.K., Davidson, W.K. and Martin, S.J. (1977): The role of defective interfering particles in persistent infection of Vero cells by measles virus. *J. Gen. Virol.* **35**, 89-97.

Rima, B.K. (1989): Comparison of amino acid sequence of the major structural proteins of the paramyxo- and morbilliviruses. In: Mahy, B.W.J. and Kolakofsky, D. (Eds.). *Genetics and pathogenicity of negative strand viruses.* Elsevier, Amsterdam. 254-263.

Rima, B.K., Earle, J.A.P., Yeo, R.P., Herlihy, L., Baczko, K., ter Meulen, V., Carabano, J., Caballero, M., Celma, M.L. and Fernandez-Munoz, R. (1995a): Temporal and geographical distribution of measles virus genotypes. *J. Gen. Virol.* **76**, 1173-1180.

Rima, B.K., Earle, J.A.P, Baczko, K., Rota, P.A. and Bellini, W.J. (1995b): Measles virus strains variations. In: ter Meulen, V. and Billeter, M.A. (Eds) Measles Virus: *Current Topics in Microbiology and Immunology.* **191**, 65-83.

Rindler, M.J., Ivanov, I.E., Plesken, H., Rodriguez-Boulan, E.J. and Sabatini, D.D. (1984): Viral glycoproteins destined for apical or basolateral membrane domains traverse the same golgi apparatus during their intracellular transport in Madin-Darby canine kidney cells. *J. Cell. Biol.* **98**, 1304-1319.

Robbins, S.J. (1983): Progressive invasion of cell nuclei by measles virus in persistently infected human cells. *J. Gen. Virol.* **64**, 2335-2338.

Rose, J.W., Bellini, W.J., McFarlin, D.E. and McFarland, H.F. (1984): Human cellular immune response to measles virus polypeptides. *Virol.* **49**, 988-991.

Rossmanith, W., Tullo, A., Potuschak, T., Karwan, R. and Sbisa, E. (1995): Human mitochondrial tRNA processing. *J. Biol. Chem.* **270**, 12885-12891.

Rota, J., Hummel, K.B., Rota, P. and Bellini, W.J. (1992): Genetic variability of the glycoprotein genes of current wild-type measles virus isolates. *Virol.* **188**, 135-142.

Rota, J.S., Wang, Z., Rota, P.A. and Bellini, W.J. (1994a): Comparison of sequence of the H, F and NP genes of measles virus vaccine strains. *Virus Res.* **31**, 317-330.

Rota, P.A., Bloom, A.E., Vanchiere, J.A. and Bellini, W.J. (1994b): Evolution of the nucleoprotein and matrix genes of wild-type strains of measles virus isolated from recent epidemics. *Virol.* **198**, 724-730.

Rozenblatt, S., Eizenberg, O., Ben-Levy, R., Lowie, V. and Bellini, W.J. (1985): Sequence homology within the morbilliviruses. *J. Virol.* **53**, 684-690.

Ruckle, G. and Roger, K.D. (1956): Studies with measles virus II: isolation of virus and immunological studies in persons who have had natural disease. *J. Immunol.* **78**, 341-355.

Rueda, P., Garcia-Barreno, B. and Melero, J.A. (1994): Loss of conserved cysteine residues in the attachment (G) glycoprotein of two human respiratory syncytial virus escape mutants that contain multiple A-G substitutions (hypermutations). *Virol.* **198**, 653-663.

Saffhill, R., Margison, G.P. and O'Connor, P.J. (1985): Mechanisms of carcinogenesis induced by alkylating agents. *Biochimica et Biophysica Acta.* **823**, 111-145.

Sanderson, C.M., McQueen, N.L. and Nayak, D.P. (1993): Sendai virus assembly: matrix protein binds to viral glycoproteins in transit through the secretary pathway. *J. Virol.* **67**, 651-663.

Sanger, F., Nicklen, S. and Coulson, A.R. (1977): DNA sequencing with chain-terminating inhibitors. *PNAS. USA.* **74**, 5463-5467.

Sato, T.A., Kohama, T. and Sugiura, A. (1988): Intracellular processing of measles virus fusion protein. *Arch. Virol.* **98**, 39-50.

Satake, M. and Ventakesan, S. (1984): Nucleotide sequence of the gene encoding respiratory syncytial virus matrix protein. *J. Virol.* **50**, 92-99.

Schluederberg, A. and Chavanich, S. (1974): The role of the nucleus in measles virus replication. *Med. Microbiol. Immunol.* **160**, 85-90.

Schmid, A., Spielhoter, P., Cattaneo, R., Baczko, K., ter Meulen, V. and Billeter, M.A. (1992): SSPE is typically characterised by alterations in the fusion protein cytoplasmic domain of the persisting measles virus. *Virol.* **188**, 910-915.

Schneider-Schaulies, S., Liebert, U.G., Baczko, K., Cattaneo, R., Billeter, M.A. and ter Meulen, V. (1989): Restriction of measles virus gene expression in acute and subacute encephalitis of Lewis rats. *Virol.* **171**, 525-534.

Schneider-Schaulies, S., Liebert, U.G., Segev, Y., Rager-Zisman, B., Wolfson, M. and ter Meulen, V. (1992): Antibody-dependent transcriptional regulation of measles virus in persistently infected neural cells. *J. Virol.* **66**, 5534-5541.

Schneider-Schaulies, S., Schneider-Schaulies, J., Bayer, M., Loffler, S. and ter Meulen, V. (1993): Spontaneous and differentiation-dependent regulation of measles virus gene expression in human glial cells. *J. Virol.* **67**, 3375-3383.

Schneider-Schaulies, S., Schneider-Schaulies, J., Schuster, A., Bayer, M., Pavlovic, J. and ter Meulen, V. (1994a): Cell-type specific MxA-mediated inhibition of measles virus transcription in human brain cells. *J. Virol.* **68**, 6910-6917.

Schneider-Schaulies, S., Schorr, J-J., Dunster, L.M., Schneider-Schaulies, J. and ter Meulen, V. (1994b): The role of host factors in measles virus persistence. *Sem. Virol.* **5**, 273-280.

Schneider-Schaulies, S., Schneider-Schaulies, J., Dunster, L.M. and ter Meulen, V. (1995): Measles virus gene expression in neural cells. In: ter Meulen, V. and Billeter, M.A. (Eds) Measles Virus: *Current Topics in Microbiology and Immunology.* **191**, 101-116.

Schultz, T.F., Huad, J.G., Whiby, D., Tizard, E.J., Dillon, M.J. and Weiss, R.A. (1992): A measles isolate from a child with Kawasaki disease: sequence comparison with contemporaneous isolates from 'classical' cases. *J. Gen. Virol.* **73**, 1581-1586.

Sechoy, O., Philippet, J.R. and Bienvenue, A. (1987): Fusion protein-fusion protein interaction within the Sendai virus identified by native bonding or chemical cross-linking. *J. Biol. Chem.* **262**, 11519-11523.

Segev, Y., Rager-Zisman, B., Isakov, N., Schneider-Schaulies, S., ter Meulen, V., Udem, S., Segal, S. and Wolfson, M. (1994): Reversal of measles virus-mediated increase of phosphorylating activity in persistently infected mouse neuroblastoma cells by anti-measles virus antibodies. *J. Gen. Virol.* **75**, 819-827.

Segev, Y., Ofir, R., Salzberg, S., Heller, A., Weinstein, Y., Isakov, N., Udem, S., Wolfson, M. and Rager-Zisman, B. (1995): Tyrosine phosphorylation of measles virus nucleocapsid protein in persistently infected neuroblastoma cells. *J. Virol.* **69**, 2480-2485.

Sheshberadaran, H., Chen, S.N. and Norrby, E. (1983): Monoclonal antibodies against 5 structural components of measles virus I: characterisation of antigenic determinants on nine strains of measles virus. *Virol.* **128**, 341-353.

Sheshberadaran, H., Norrby, E., McCullough, K.C., Carpenter, W.C. and Orvel, C. (1986): The antigenic relationship between measles virus, canine distemper virus and rinderpest virus studied with monoclonal antibodies. *J. Gen. Virol.* **67**, 1381-1392.

Shiozawa, S., Yoshikawa, N., Iijima, K. and Negishi, K. (1988): A sensitive radioimmunoassay for circulating α-IFN in the plasma of healthy children and patients with measles virus infection. *Clin. Exp. Immunol.* **74**, 366-369.

Sidhu, M., Menonna, J.P., Cook, S.D., Dowling, P.C. and Udem, S.A. (1993): Canine distemper virus L gene: sequence and comparisons with related viruses. *Virol.* **193**, 50-65.

Sidhu, M.S., Crowley, J., Lowenthal, A., Karcher, D., Menonna, J., Cook, S., Udem, S. and Dowling, P. (1994): Defective measles virus in human SSPE brain. *Virol.* **202**, 631-641.

Spriggs, M.K. and Collins, P. (1986): Sequence analysis of the P and C protein genes of human parainfluenza virus type 3: pattern of amino acid sequence homology among paramyxovirus proteins. *J. Gen. Virol.* **67**, 2705-2719.

Spriggs, M.K., Olmsted, R.A., Venkatesan, S., Coligan, J.E. and Collins, P. (1986): Fusion protein of human parainfluenza virus type 3: nucleotide sequence of the gene, direct identification of the cleavage activation site and comparison with other paramyxoviruses. *Virol.* **152**, 241-251.

Stec, D.S., Hill, M.G. and Collins, P.L. (1991): Sequence analysis of the polymerase L gene of human respiratory syncytial virus and predicted phylogeny of non-segmented negative-strand viruses. *Virol.* **183**, 273-287.

Stephenson, J.R. and ter Meulen, V. (1979): Antigenic relationships between measles virus and canine distemper virus: comparison of the immune response in animals and humans to individual virus-specific polypeptides. *Proc. Natl. Acad. Sci. USA.* **76**, 6601-6605.

Suryanarayana, K., Baczko, K., ter Meulen, V. and Wagner, R. (1994): Transcription inhibition and other properties of matrix proteins expressed by matrix gene clones from measles virus and diseased human brain tissue. *J. Virol.* **68**, 1532-1543.

Tamin, A. Rota, P.A., Wang, Z., Heath, J.L., Anderson, L.J. and Bellini, W.J. (1994): Antigenic analysis of current wild-type and vaccine strains of measles virus. *J. Infect. Dis.* **170**, 795-801.

Taylor, M.J., Godfrey, E., Baczko, K., ter Meulen, V., Wild, T.F. and Rima, B.K. (1991a): Identification of several different lineages of measles virus. *J. Gen. Virol.* **72**, 83-88.

Taylor, J., Pincus, S., Tartaglia, J., Richardson, C., Alkhtib, G., Breidid, D., Norton, E. and Paoletti, E. (1991b): Vaccinia virus recombinants expressing either measles virus fusion or haemagglutinin glycoproteins protects dogs against CDV challenge. *J. Virol.* **65**, 4263-4274.

ter Meulen, V., Katz, M., Kackell, Y.F., Barbanti-Brodano, G., Koproswski, H. and Lennette, E.H. (1972): SSPE: *in-vitro* characterisation of viruses isolated from brain cells in culture. *J. Infect. Dis.* **126,** 11-17.

Thomas, S.M., Lamb, R.A. and Paterson, R. (1988): Two mRNAs that differ by two non-templated nucleotides encodes the amino co-terminal proteins P and V of the paramyxovirus SV5. *Cell.* **54,** 891-902.

Tsukiyama, K., Sugiyama, M., Yoshikawa, Y. and Yamanouchi, K. (1987): Molecular cloning and sequence analysis of the rinderpest virus mRNA encoding the haemagglutinin protein. *Virol.* **160,** 48-54.

Tsukiyama, K., Yoshikawa, Y. and Yamanouchi, K. (1988): Fusion glycoprotein (F) if the rinderpest virus: entire nucleotide sequence of the F mRNA and several features of the F protein. *Virol.* **164,** 523-530.

Udem. S.A. and Cook, K.A. (1984): Isolation and characterisation of measles virus intracellular nucleocapsid RNA. *J. Virol.* **49,** 57-65.

Ueda, S., Okuno, Y., Hamamoto, Y. and Chen, H. (1975): SSPE: isolation of a defective variant of measles virus from brain obtained at autopsy. *Biken J.* **18,** 113-122.

Vainionpaa, R., Hyypia, T. and Akermann, K.E.O. (1991): Early signal transduction of measles virus infected lymphocytes is unaltered by second messengers activate virus replication. *J. Virol.* **65**, 6743-6748.

Vanchiere, J.A., Bellini, W.J. and Moyer, S.A. (1995): Hypermutation of the phosphoprotein gene altered mRNA editing in the Hamster neurotropic strain of measles virus. *Virol.* **207**, 555-561.

Van Binnendijk, R.S., Poelen M.C.M., deVries, P., Voorma, H. O., Osterhaus, A.D. and Uytdehaag, F.G. (1989): Measles virus specific human T cell clones. Characterisation of specificity and function of CD4 helper/cytotoxic and CD8+ cytotoxic clones. *J. Immunol.* **142**, 2847-2854.

Van Binnendijk, R.S., Poelen, M.C.M., Kuipers K.C., Osterhaus, A.D.M.E. and Uytdehaag, F.G.C.M. (1990): The predominance of CD8+ T cells after infection with measles virus suggest a role for CD8+ class I MHC-restricted cytotoxic T lymphocytes (CTL) in recovery from measles. Clonal analyses of human CD8+ class I MHC-restricted CTL. *J. Immunol.* **144**, 2394-2399.

van Binnedndijk, R.S., van Baalen, C.A. and Poelen, M.C.M. (1992): Measles virus transmembrane fusion protein synthesised *de novo* or presented in immunostimulating complexes is endogenously processed for HLA class I and class II restricted cytotoxic T cell recognition. *J. Exp. Med.* **176**, 119-128.

Varior-Krishnan, G., Trescol-Biemont, M.C., Naniche, D., Rabourdin-Combe, C. and Gerlier, D. (1994): Glycosyl-phosphatidylinositol-anchored and transmembrane forms of CD46 display similar measles virus receptor properties: virus binding, fusion and replication; down-regulation by haemagglutinin; virus uptake and endocytosis for antigen presentation by MHC class II molecules. *J. Virol.* **68**, 7891-7899.

Varsangai, T.M.H., Jornval, H. and Norrby, E. (1985): Isolation and characterisation of the measles virus F1 polypeptide: comparison with other paramyxovirus fusion proteins. *Virol.* **147**, 110-117.

Vartanian, J-P., Meyerhans, A., Sala, M. and Wain-Hobson, S. (1994): G-A hypermutation of the human immunodeficiency virus type I genome: evidence for dCTP pool imbalance during reverse transcription. *PNAS USA.* **91**, 3092-3096.

Vidal, S., Curran, J. Orvell, C. and Kolakofsky, D. (1988): Mapping of monoclonal antibodies to the Sendai virus phosphoprotein and the location of its phosphates. *J. Virol.* **62**, 2200-2203.

Vidal, S., Curran, J. and Kolakofsky, D. (1990a): A stuttering model for paramyxovirus phosphoprotein mRNA editing. *EMBO.* **9**, 2017-2022.

Vidal, S., Curran, J. and Kolakofsky, D. (1990b): Editing of the Sendai virus P/C mRNA by G insertion occurs during mRNA synthesis via a virus encoded activity. *J. Virol.* **64**, 239-246.

de Vries, P., van Binnendijk, R.S., van der Marel, P., van Wezel, A.L., Voorma, H.U., Sundquist, B., Uytdehaag, F.G.C.M. and Osterhaus, A.D.M.E. (1988): Measles virus fusion protein presented in immune-stimulating complex (ISCOM) induces haemolysis-inhibiting and fusion-inhibiting antibodies, virus specific T cells and protection in mice. *J. Gen. Virol.* **69**, 549-559.

Wagner, R.W. and Nishikura, K. (1988): Cell cycle expression of RNA duplex unwindase activity in mammalian cells. *Mol. Cell. Biol.* **8**, 770-777.

Wagner, R.W., Yoo, C., Wrabetz, L., Kamholz, J., Buchalter, J., Hassan, N., Khalili, K., Kim, S.U., Perussia, B., McMorris, F.A. and Nishikura, K. (1990): Double-stranded RNA unwinding and modifying activity is detected ubiquitously in primary tissues and cell lines. *Mol. Cell. Biol.* **10**, 5586-5590.

Ward, B.J., Johnson, R.T. *et al.*. (1989): Spontaneous proliferation of peripheral mononuclear cells in natural measles virus infection. *Clin. Immunol.* **61**, 236-248.

Wechsler, S.L., Weiner, H.L and Fields, B.N. (1979):Immune response in subacute sclerosing panencephalitis: reduced antibody response to the matrix protein of measles virus. *J. Immunol.* **123**, 884-889.

Weiss, R. (1992): Measles battle loses potent weapon. *Science.* **258**, 546-547.

Wild, T.F., Malvoisin, E. and Buckland, R. (1991): Measles virus: both the haemagglutinin and the fusion glycoproteins are required for fusion. *J. Gen. Virol.* **72**, 439-442.

Wild, T.F., Bernard, A., Spehner, D. and Drillien, R. (1992): Construction of vaccinia virus recombinants expressing several measles virus proteins and analysis of their efficacy in vaccination of mice. *J. Gen. Virol.* **73**, 359-267.

Wild, T.F., Fayolle, J., Beauverger, P. and Buckland, R. (1994): Measles virus fusion: role of the cysteine-rich region of the fusion glycoprotein. *J. Virol.* **68**, 7546-7548.

Wong, T.C. and Hirano, A. (1987): Structure function of bicistronic RNA encoding the phosphoprotein and matrix protein of measles virus. *J. Virol.* **61**, 584-589.

Wong, T.C., Wipf, G. and Hirano, A. (1987): The measles virus matrix gene and gene product defined by *in-vitro* and *in-vivo* expression. *Virol.* **157**, 497-508.

Wong, T.C., Ayata, M., Hirano, A., Yoshikawa, Y., Tsuruoka, H. and Yamanouchi, K. (1989): Generalised and localised biased hypermutations affecting the matrix gene of measles virus strain that causes SSPE. *J. Virol.* **63**, 5464-5468.

Wong, T.C., Ayata, M., Ueda, S. and Hirano, A. (1991): Role of biased hypermutation in the evolution of SSPE virus from progenitor acute measles virus. *J. Virol.* **65**, 2191-2199.

Yoshikawa, Y. and Yamanouchi, K. (1984): Effects of papaverine treatment on replication of measles virus in human neural and ono-neural cells. *J. Virol.* **50**, 489-496.

Yoshikawa, Y., Mizumoto, K. and Yamanouchi, K. (1986): Characterisation of messenger RNA's of measles virus. *J. Virol.* **67**, 2807-2812.

Yoshikawa, Y., Tsuroka, H., Matsumoto, M., Haga, T., Shioda, T., Shibuta, H., Sato, T.A., and Yamanouchi, K. (1990): Molecular analysis of structural protein genes of the Yamagata-1 strain of defective subacute sclerosing panencephalitis. II: Nucleotide sequence of cDNA corresponding to the P plus M dicistronic mRNA. *Virus Genes*. **4**, 151-162.

Yu, Q., Davis, P.J., Brown, T.D.K. and Cavanagh, D. (1992a): Sequence and *in-vitro* expression of the M2 gene of turkey rhinotracheitis virus. *J. Gen. Virol.* **73**, 1335-1363.

Yu, Q., Davis, P.J., Li, J. and Cavanagh, D. (1992b): Cloning and sequencing of the matrix protein gene of turkey rhinotracheitis virus reveal a gene order different from that of respiratory syncytial virus. *Virol.* **186**, 426-434.

Zerial, M., Huylebroeck, D. and Garoff, H. (1987): Foreign transmembrane peptides replacing the internal signal sequence of transferin receptor allows its replication and membrane binding. *Cell.* **48**, 147-155.